THE EFFECTS OF KRAFT MILL EFFLUENTS ON NYMPHS OF *CALLIBAETIS* SP. (EPHEMEROPTERA) AND *ACRONEURIA PACIFICA* (PLECOPTERA)

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INTRODUCTION

This paper is written as a record of experiments undertaken during the fall of 1948 and spring of 1949 at Oregon State College to determine the effects of some of the various Kraft mills waste products on aquatic insect nymphs of the orders Ephemeroptera and Plecoptera. The study reported on herein is one of a number of similar projects undertaken recently by the Department of Fish and Game Management, Oregon State College. These studies have included research on the effects of Kraft mills effluents on king salmon, silver salmon, and cutthroat trout.

In the United States people are beginning to realize to a more and more satisfying degree, the importance of fish and wildlife as natural resources of great value. Although much has been done along the channels of conservation, sustained production of wild animals, pollution control, artificial propagation and rehabilitation of habitat for the benefit of these natural resources, a great deal more has still yet to be accomplished. Of the fields of endeavor, pollution control is uppermost in the minds and activities of an increasing number of fisheries, biologists, and conservationists. In some cases industry is vitally interested in pollution control as evidenced by

the cooperation of the National Council of Stream Improvement, Incorporated, an agency of the Pulp, Paper, and Paperboard Industries, in sponsoring the studies involving the effects of Kraft mills effluents on salmonoid fishes.

State and National legislative bodies are formulating and assembling their plans of attack against pollution. The laws attempt to regulate such factors as the types and amounts of the different sewages and effluents that may be discharged into any body of water containing economically important aquatic forms of life or waters in which the public may commonly come into contact. These laws will and do affect the various industries that utilize to some extent the waters of any particular area for part of their operational set-up. Many of the various Kraft mills and other industrial plants are located on or near bodies of water, into which they discharge the waste products of their manufacturing. There is an increasing demand upon these waters for such function.

Most sportsmen automatically assume that the discharge of effluents from sources such as above will be detrimental to fish and fishing. These same sportsmen will often band together to initiate laws and regulations to curb discharge of waste effluents or to force industrial administrators to provide ways and means of treating these wastes before their discharge. Companies that are forced to make changes in this manner are faced with

additional expenditure, material shortages, short-comings of designs, and time limitations.

Would it not seem fair then, in view of the facts, that before such laws are put into effect that some provision be made to accurately determine the effects of the wastes in question on the different aquatic forms of life that are important in the relationship between animals and pollution?

In nature there are two general ways by which an animal may be exterminated. First, the animal may be killed, directly, by one means or another. Secondly, the animal may die as a result of deprivation of the necessary food, shelter or warmth. Pollution in its various forms has been known to kill fish directly, but frequently underemphasized have been the effects of toxic substances on the living organisms that provide a large part of the food of fishes.

Is it known, however, in the illustration above, what concentrations of the pollutant materials will be fatal to the fish, or that the main source of aquatic food will be destroyed? In most cases, these essential pieces necessary to make a complete picture are not known.

It is the purpose of this study, therefore, to make a step in the direction of clearing up some of these questions, namely:

1. At what concentrations will certain of the pollutant materials be lethal to aquatic insects?

2. Are aquatic insects more or less resistant to certain forms of pollution than are their consumers, the salmonoid fishes?

A vast field of study is opened up in these questions. This research and report may serve no other purpose than to open the way for more strenuous efforts and significant findings toward the solution of the problems.

Aquatic insects of the orders Ephemeroptera (mayflies) and Plecoptera (stoneflies) were used in this study. The chemical compounds used in the testing were limited to compounds known to be present in Kraft mills effluents. Approximate minimum lethal concentrations of these chemicals in solution on one species of Ephemeroptera and one species of Plecoptera were determined.

These insects were chosen as test animals because of their importance as young salmon and trout foods in fresh water and also because of their abundance in the lakes and streams of the area in which the tests were conducted. According to Dimick and Mote (1934) "these insects (Ephemeroptera) form the first choice of all cutthroat and rainbow trout in Oregon, judged by the frequency and quantity of their occurence in the fish stomaches analyzed. Mayflies, both aquatic and aerial types, formed 26.5 percent of all food found in the stomaches of cutthroat trout examined. In the rainbow trout, ranging from four to 19 inches in length, taken from streams from February to

September inclusive, mayflies were the predominant food organisms except in fish more than 15 inches in length. They formed 47.1 percent of all food consumed. Stoneflies, both adults and nymphs, ranked second in the diet of rainbow trout examined from Oregon streams. Adult stoneflies composed 15.03 percent and nymphs composed 1.04 percent of the total foods found in rainbow trout from streams."

Wallis (1948) found that aquatic insects formed about 75 percent of the total food consumed by brook, rainbow, and brown trout in Crater Lake National Park, Oregon. In order of their importance as food, the six most important aquatic insects were: caddis-fly larvae, diptera larvae and pupae, diptera adults, mayfly nymphs, stonefly nymphs and mayfly adults.

Webster (1944) stated that the aquatic food of brown trout from a creek in New York was made up of about 90 percent mayfly nymphs. From a study made by Ide (1942) in Canada, the diet of Salvelinus fontinalis was made up of 64 percent aquatic organisms. Of this amount, 24 percent was of emerging forms of insects such as Ephemeroptera and Plecoptera.

The foregoing citations stand as evidence of the importance of mayflies and stoneflies in the food habits of some of the important salmonoid fishes in fresh water. It is assumed that the use of these insect groups is justified in this study.

HISTORICAL ASPECTS

"Up to about 1855, the question of depletion of the shellfish in the vicinity of New York rested mainly upon over-dredging by fishermen and the destruction of the natural enemies of shellfish but, about this time, manufacturing began to spring up in the district along New York harbor and the wastes from these plants, finding its way into the streams, commenced to take its toll from this industry." (Report of Interstate Sanitation Commission of New York City, 1946).

The above citation is a description of the probable earliest beginnings of pollution in this country. From this date on, pollution became an increasingly more acute problem for those interested in conservation of natural resources and public health.

Pollution on the West coast of the United States had a much later start because of the later settlement of that area and slower development of industry there. However, about 1900 industrial pollution became an important problem.

Many important advances have been made in recent years in the field of sewage disposal and pollution control. More and more cities have installed sewage treatment plants. Through improved methods and more efficient utilization of waste products, the manufacturing industry

in specific instances has been able to reduce the amounts of toxic effluents being cast into the lakes and streams. The Institute of Paper Chemistry at Appleton, Wisconsin, an educational and research institution of the Pulp, Paper and Paperboard Industries has undertaken studies of the effects of Kraft mills effluents on Daphnia and a species of minnow. The research reported on herein was based to some extent upon the results of this study by the institute.

According to a general survey of the literature on the experimental work done on the effects of pollution on aquatic life, little has been done in the United States using insects as test animals. Stickney (1922) has made a study of the relation between dragonfly nymphs, temperature, and acids. Most of the other investigators in the United States such as Stiemke, Wells, Ellis, and Powers have performed experiments mainly on fish. It is interesting to note that the Scandinavian and European countries have also been active in this type of research. Wuhrmann, of Switzerland, and Vallin, of Sweden, are among those who have made important contributions to the understanding of pollution in relation to fish.

A large number of studies have been made on the effect of DDT and many other insecticides on the non-aquatic and aerial stages of aquatic insects. Little of this work is closely related to the problem considered in this report.

COLLECTION, HOLDING, AND IDENTIFICATION OF TEST ANIMALS

The best methods of collection and holding of the test animals were discovered by repeated trials of the various procedures that may be used. Identification of species was ordinarily made at the laboratory after the individuals were collected and separated according to gross characteristics.

Mayfly nymphs

Early in the summer in Western Oregon, mayfly nymphs begin to appear in greater and greater numbers. First in the larger streams and ponds, then later in practically all standing water including many small overflow pools and ditches containing a few gallons of water. The nymphs may occur in tremendous numbers throughout the remainder of the summer and the fall. It is during this time that the collection of nymphs is most easily accomplished. It follows that this would be the most opportune time to begin a study involving these aquatic insects. In this study, large numbers of the nymphs were used, and since they were of a single species, considerable time was used in collection activities. Actually, the larger the number of test animals used in any one test will correspondingly increase the accuracy of the results of that test.

During the biological assays conducted in this study,

several thousand manyfly nymphs were used. Ordinarily many more would have been used but the investigation was begun during the fall season when specimens were becoming less and less abundant.

Collection of mayfly nymphs proved to be very simple indeed while they were at their peak of abundance. An ordinary long-handled aquatic insect net was very satisfactory. The diameter of the net was 12 inches. The mesh of the net material rather coarse to prevent the filling of the bag too rapidly by the large quantities of mud and debris that was picked up as the net was drawn along the bottom of the pool after the nymphs. After making a sweep with the net, the entire mass of insects, mud and debris was washed off into a container; the insects were separated from the foreign material later at the laboratory.

The collecting container used was a large-mouthed, five-gallon, glass jug with handle. The glass container was used because it facilitated estimation of the extent of the collection up to any point. Also, the container was light-weight enough to be easily carried. It was found that if the jug was filled completely with water, washing of the insects and debris from the net after sweeping the bottom of the pool was made easier and in the trip to the laboratory there would be less agitation of the water and consequently less disturbance of the captured insects.

After being collected in the field, the nymphs were brought to the laboratory and separated from the debris on a large, white porcelain pan. First the nymphs were partly separated from the debris by pouring off the supernatant water from the jug. About half of the nymphs were obtained from the container in this manner. After being poured from the jug, the water was passed through a wire screening of mesh 18 by 14 which collected the individual nymphs. As they were caught on the screen, the nymphs were picked off one by one with a pair of thumb forceps and placed into the stock containers. After this was accomplished, pieces of vegetation and debris were lifted out of the collecting jug and placed in a dry, flat, white porcelain pan and then the remainder of the nymphs were picked out and placed with the others.

A small pair of forceps proved to be very satisfactory for the handling of the nymphs. Although the mayfly nymphs were hardy, care was taken not to injure any specimens by unduly rough handling.

Several hundred nymphs were collected on each trip to the field, providing sufficient test animals to conduct 25 to 30 tests. The nymphs collected were allowed to acclimatize themselves to the conditions in the laboratory for a period of one or two weeks before being used in experiments. The stock containers were kept clear of all debris and dead specimens to prevent fouling of the water.

The species of mayfly used in the study were those of pond origin; stream dwelling types being presumably less adaptable to laboratory conditions. This mayfly, Callibaetis Eaton, was determined by George F. Edmunds of the University of Utah. After subsequent collecting trips, the nymphs captured were identified by the use of the keys from Needham (1935).

Early in the project, about 50 nymphs of a species in the family Heptageniidae were collected from a rocky stream and placed in a glass container, 10 inches wide and three inches deep, holding one liter of tap water. The temperatures maintained in the container were approximately those of the stream from which the larvae were taken. All of the specimens had died within 48 hours of the time they had been removed from the stream. This species was discarded as a possibility for test animals.

Stonefly nymphs

Plecopteran nymphs present a somewhat different problem in collection and holding than do mayfly nymphs. They were never found to be in as great abundance as were the mayfly nymphs at some seasons. Apparently their relative numbers do not vary greatly from season to season over the year. Their habitat is usually under rocks and among the detritus of a swiftly flowing stream. As with Ephemeroptera the collecting equipment used was very simple,

consisting only of an aquatic net and a five-gallon holding jug. The aquatic net was modified in shape somewhat by bending the circular rim of the net into a half-moon shape. The straight side being away from and perpendicular to the long axis of the handle.

To capture stonefly nymphs, the operator placed himself in likely-looking, shallow, rocky riffles of streams. Then by facing downstream and by placing the net upright into the water with the bottom straight edge on the bottom of the riffle and with the handle held at arms length downstream, the collector was ready to begin collecting. Moving slowly downstream, keeping the net in the correct position ahead of him, the collector dislodged all the rocks possible and as the current caught the disturbed nymphs they were washed into the waiting net. When the length of the riffle had been covered in this manner, the net was brought ashore where the nymphs were picked out by hand and placed into the collecting jug. Because of the nymphs' habit of hanging tenaciously onto objects such as the net material or vegetation in the net, thumb forceps could not be employed in their handling; the nymphs often were injured by the forceps before they would lose their hold on the object. Fingers proved to be the most satisfactory tool with which to handle the nymphs.

Stonefly nymphs of the species used are very active and unless they are provided with something to which they

can cling they will run continuously around in their container or will gather together in clumps of five or six, all of which does not promote higher survival rates. Because of this, a large clump of clean grass roots were usually placed into the collecting jug to provide them with a support. After being transported back to the laboratory, the root clump was removed and placed into a clean, white pan. Ordinarily, most of the nymphs remained attached to the roots during the transfer.

It was found satisfactory to use either grass root clumps or rubber stoppers for the nymphs to cling to while in the stock container. Upon being placed into the container, the nymphs were not long in beginning the "push-up" type of respiratory movements with their bodies. At this time it was assumed that the dissolved oxygen content of the water was dropping too low for the nymphs to be content. Upon aeration of the water with a small, electric Thiberg aerator, the respiratory movements became less violent and less continuous. The stock waters were aerated at all times, although this was apparently not absolutely necessary judging from the trials described earlier. Probably where large numbers of nymphs are to be kept together for long periods, aeration is essential for their well-being.

During the first collections, all species of stonefly nymphs were captured and brought to the laboratory. Three

species predominated in numbers and the selection of the test species was narrowed down to these. As they were collected, the three species were separated and placed in containers under similar conditions. The different species were identified by the use of the keys developed by Claasen (1931). Thirty individuals of each species were placed into glass bowls, 10 inches wide and three inches, deep, containing tap water. The results were observed. The Nemoura cinctipes Banks would not survive longer than one or two days, many dying within a few hours of capture. The Nemoura oregonensis Claasen were hardier and most would survive three to five days; practically all would live through the first two days. The Acroneuria pacifica Banks proved rather well adapted to the conditions and approximately 75% of them survived for a period of two weeks. However, up to the end of that period, one or two individuals succumbed almost each day. Even with aeration after that period, one or two would die each week. Acroneuria pacifica Banks was chosen as the test species. The determination of species of the various nymphs was made by Mr. Stanley G. Jewett, Jr., Aquatic Biologist, United States Fish and Wildlife Service.

PROCEDURE AND SIGNIFICANCE OF METHODS

General Considerations

For the most part, the procedures set forth by Hart, Doudoroff and Greenbank (1945) were followed wherever possible and practicable. These procedures were originally formulated for use in investigations using fish, primarily, as test animals, however, it seems reasonable to assume that the so-called "standard" procedures set forth by them should also be used in experiments of the same nature involving aquatic insects.

The toxic components of Kraft mill wastes have been determined. It is from these components that the compounds used in the testing in this study were chosen. The test solutions used were prepared in accordance with the instructions found in a number of general chemistry text books and publications dealing with the qualitative examination of water such as Standard Methods for the Examination of Water and Sewage (1946) and McPherson (1933). The actual compounds used were chemically pure (C.P.) chemicals procured from scientific supply companies.

In this discussion the principle physiological effects of the chemical solutions under consideration, the general characteristics, and their preparation will be discussed. A general description of the calculations involved are also covered.

Solutions and Their Preparation

1. Hydrogen sulfide (H_2S)

According to King (1947) hydrogen sulfide is a colorless gas at ordinary temperatures. It is not highly soluble in water; one liter of water will dissolve about three liters of the gas at ordinary temperatures. In the reaction with water, hydrogen sulfide forms a solution with acid properties:

$$H_2S + 2H_2O \rightleftharpoons 2\,H_3O^+ + S^{--}$$

Solutions containing hydrogen sulfide were prepared by heating a capsule of paraffin and sulfur and bubbling the resultant gas into a flask containing distilled water. The exact normality of this solution was then determined potentiometrically by a method described in part by Van Horn (1944). After the normality had been determined, the strength of the solution in parts of hydrogen sulfide per million parts of water was calculated by the following method:

$$\text{Parts per million (p.p.m.)} = \text{normality} \times \text{equivalent weight} \times 1000$$

The amount of solution used in the test containers was calculated as follows:

$$\frac{\text{p.p.m. of stock solution}}{\text{p.p.m. desired for test solution}} = \frac{\text{Desired Volume of test solution}}{\text{Volume of stock solution to use.}}$$

Hydrogen sulfide is a very toxic gas. King states that the physiological effect of this compound on animals

i s the paralysis of the nerve centers of the heart and lungs.

2. Sodium sulfide (Na_2S)

According to Briscoe (1945), sodium sulfide in aqueous solutions is strongly alkaline because of hydrolysis of the salt:

$$(2\ Na^+ + S^=) + HOH \rightarrow (Na^+ + HS^-) + (Na^+ + OH^-)$$
$$(Na^+ + HS^-) + HOH \rightarrow H_2S + (Na^+ + OH^-)$$

Van Horn (1946), states that in a sodium sulfide solution, the sulfide ion undergoes hydrolysis, thus:

$$S^= + HOH \rightleftharpoons HS^- + OH^-$$

In this state, the sulfur is present in the form of the hydrosulfide and the reaction would probably continue as follows:

$$HS^- + HOH \rightleftharpoons H_2S + OH^-$$

The fact that the hydrogen sulfide would be dissipated from the water at a rapid rate would unbalance the equilibrium with the result that the reaction would take place continuously to the right. Under these circumstances the actual toxic affect would be caused to a greater extent by hydrogen sulfide than by either sodium sulfide or sodium hydrosulfide.

Jones (1948), found that a sodium sulfide solution of .001 normal (39 parts per million), brought to a pH of 6.8 with sulfuric acid caused an immediate negative reaction to the stickleback, Gasterosteus sp.

A normal solution is one that contains one equivalent weight in grams, of a compound, in one liter of water. A normal solution of sodium hydroxide was prepared by adding 39 grams of the sulfide to a graduated cylinder and then by filling the cylinder to one liter with distilled water. The exact normality of this stock solution was then checked potentiometrically and as in the case of all the chemicals the test solutions were prepared by dilution of the stock solution.

3. Sodium Hydroxide (NaOH)

Sodium hydroxide is a white, crystalline, brittle solid. MePherson (1933) says that it is a very corrosive substance and has a strong disintegrating action upon both animal and vegetable tissue. It is extremely soluble in water, liberating a great deal of heat. Large quantities of this compound are used in preparing wood pulp from which paper is made. Sutermeister (1946) states that "the cooking or "white liquor" used in the sulfate process (process for preparing wood pulp for paper products) has as its principle active constituents sodium hydroxide and sulfide . . . . . ." These liquors are commonly part of the waste effluents of Kraft mills using the sulfate process.

Sodium hydroxide is classed as a strong base and is often called alkali. The exact normality of the solutions made up were determined by titration with sulfuric acid and methyl orange indicator.

4. Sodium Carbonate (Na_2CO_3)

This compound is a white solid at ordinary temperatures. Because of hydrolysis forming a weak acid and hydroxyl ions, sodium carbonate exhibits a distinoly basic reaction in aqueous solution:

$$Na_2CO_3 + H_2O \rightleftharpoons 2Na^+ + OH^- + HCO_3^-$$

This accounts for the high pH as is evident from Table 3.

The exact normality of the test solutions prepared was determined by titration with sulfuric acid using methyl orange indicator. Frequent checks were made on the strength of the various solutions. The stock containers were stored under conditions of temperature identical to the temperatures maintained during the actual testing of the insect larvae.

5. Sodium Sulfate (Na_2SO_4)

Sodium sulfate is a white solid that does not appreciably alter the pH of an aqueous solution of that salt. For that reason the stock solutions were prepared by weighing out the exact amount of the sulfate to make a one normal solution. No means was available to check the precise normality of the solutions prepared. This sulfate is used in the Sulfate process for preparing wood pulp for various Kraft mill products.

6. Sodium Hydrosulfide (NaHS)

What was said in general for hydrogen sulfide may be said for sodium hydrosulfide solutions. The two

compounds react similarly and have much the same characteristics. Hydrosulfide however, is a colorless solid while hydrogen sulfide is present in the gaseous state at ordinary temperatures. Solutions of this compound were prepared by passing hydrogen sulfide through a sodium hydroxide solution. The exact normality of the solution was then determined by use of the Beckman pH meter.

Water Supply

In preparing the testing solutions, tap water was used. This water was obtained from the same general source from which the test animals were obtained, the municipal reservoir of the City of Corvallis, Oregon. It was from this reservoir that most of the Ephemeroptera nymphs used as test animals were obtained. Part of the Plecoptera used were collected in the outlet stream of the reservoir. In this situation the closest relationship possible between the actual habitat of the test animals and the testing solutions was made possible.

In a qualitative analysis, the water was found to possess the following constituents of the values indicated. These values are compared below to the findings of Ellis (1937) for waters that support good mixed fish faunas in the United States.

Quality or Constituent	Value for Test Water	Findings by Ellis for 90 percent of waters tested
pH	7.0-7.5	6.7-8.3
Dissolved oxygen	9 -12 p.p.m.	5.0-9.8
Free carbon dioxide	1.0-2.0 p.p.m.	0.1-5.0
Specific conductivity (Mhos x 10^{-6} at 25°)	1000-1200	50-1100
Chlorine (ortho tolideine)	.1 p.p.m. or less	
Methyl orange alkalinity	20-65 p.p.m. ($CaCO_3$)	
Biochemical oxygen demand	0.3-0.5 p.p.m.	

It was found that mayfly nymphs of all sizes of Callibaetis sp., will survive in a healthy condition for periods of one to two months or longer in small containers holding only a few hundred milliliters of tap water from the source described later. One hundred nymphs were kept in one liter of tap water in a glass container 10 inches wide and three inches deep, for a period of one month with only a few deaths among the lot. About one-third of these nymphs developed and emerged as sub-imagos during that time.

Trials were carried out concurrently with the above testing to determine the suitability of natural waters, and distilled waters as stock mediums. One hundred mayfly nymphs were placed in a glass container 10 inches wide and three inches deep holding two liters of freshly distilled water. All but a few of the nymphs were dead within 48 hours. Pond water from which the nymphs were obtained was subject to the same test as above. Most of the nymphs died within three or four days. About 10 percent,

however, lived on and remained in good condition for several weeks. Under the conditions, the water in this second test seemed to stagnate rapidly. Shortly after death the nymphs turned a noticeable red color in the water. It was assumed that the rapid fouling of the water was responsible in some way for the high mortality among the nymphs.

Going further, it was found that 10 nymphs would survive for at least three weeks, without visible source of food, in an ordinary Petri dish holding only 50 milliliters of the tap water. From the results of these trials, it was decided that the tap waters would be the most suitable medium to use for the stock containers.

Temperature Regulation

During the warm weather phase of this study, temperatures of the test solutions were kept within certain limits by cold water baths. The source of the water was the tap water described earlier and the temperature maintained in the baths varied with the temperature of the running tap water. Later, under winter conditions, the test solutions were raised and maintained within certain limits by electric lamps from which the heat was distributed over the containers by means of cardboard hoods. During the summer, for testing of Ephemeroptera, the temperature varied from 17 to 19 degrees centigrade in the

test containers; during the winter and for Plecoptera experiments the temperature ranged from eight to 12 degrees centigrade as is shown in the tables of the minimum lethal concentration.

Determination of Hydrogen ion Concentrations

The following paragraph was taken from a report to the Interstate Sanitation Commission of New York: (1939) "Powers(1930) in reviewing the problem (of pH variation in natural waters) summarized the existing data by saying that aquatic organisms are able to withstand a wide range in pH. The writer has confirmed this statement with grammarids, daphnia, unionids, and planaria in connection with pollution tests, as well as with goldfish, perch and catfish. It might seem, therefore, that the pH value of stream water would be of little consequence in pollution studies and in determining standards of purity for water. However, the pH of natural water is determined by substances in solution, particularly carbonates and carbon dioxide, and various salts and other materials that constitute a fair buffer system, so that water more acid than pH 7.0 or more alkaline than pH 8.5 is rarely found in our inland streams unless there be some unusual factor in the complex. Pond water, bog waters and lake waters vary over a wider range, but the combination of stream flow, aeration, and buffer substances holds the hydrogen ion

concentration outside of the limits pH 7.0 and pH 8.5 until it could be definitely shown that the deviation was due to natural causes rather than pollution through human agencies. Even rather badly polluted streams were usually within these limits and when sufficient material had added to the water to produce a hydrogen ion concentration more acid than pH 7.0 or more alkaline than pH 8.5, the buffer salts and carbonate systems were definitely disturbed and conditions harmful to fishes were usually found. The determination of pH therefore is an important aid in the study of polluted waters in spite of the range of tolerance of fishes to pH changes in unpolluted waters, because excessive variation in the hydrogen ion concentration is indicative of harmful changes in the complex of dissolved substances normally found in river waters."

Chapman (1931) has said that "the hydrogen ion concentration of a given environment may be looked upon as sort of a "symptom" rather than a "cause" of any particular condition in the environment."

The above statements generally evaluate the importance of pH in a study of this type. The pH of the test solutions was determined colorimetrically with LaMotte Indicator and by the use of a Beckman pH meter. Determinations were made before and after each significant test to ascertain changes undergone during the test.

In numerous determinations of pH of the water of the reservoir and of the outlet stream from which most of the test animals were collected, the values ranged from pH 7.0 to 7.5 during the period of study.

As to the effects of pH on animals, Chapman states that it was found that an addition of 0.00004 percent solution of hydrochloric acid in the circulating blood of a frog is sufficient to kill the heart. The respiratory center and other tissues are apparently just as susceptible to changes in hydrogen ion concentrations.

Stickney (1922) in experimenting with the dragonfly, Libellula pulchella, found that it was indifferent to acidity even to a pH of 1.0, and lived apparently unharmed in various pH values.

To repeat what has already been said, the pH of the test solutions was taken to indicate, more than its toxicity to animals, the nature or strength of the chemical pollutant involved.

The Determination of Dissolved Oxygen Content and the Oxygen Consumption of Ephemeroptera Nymphs.

Plecoptera nymphs have been found living in waters with as little as 5.2 p.p.m. of dissolved oxygen and mayfly nymphs were collected in waters containing only 5.8 p.p.m., Dimick (1945). For the purposes of this study it was assumed that the p.p.m. of dissolved oxygen would

not be an important factor in the affects of the toxic chemicals upon these two groups of insects if the content remained safely above 5.8 p.p.m. Ordinarily the dissolved oxygen content in the test solutions remained well over 8.0 p.p.m.

The dissolved oxygen content of the test solutions was determined by the unmodified Winkler method as described in Theroux (1943) and in Standard Methods for the Examination of Water and Sewage (1946). In view of the fact that the chemicals used as biological assay materials are not organic substances, it follows that the biochemical oxygen demand (B.O.D.) of a solution of these chemicals would not necessarily be high. From the tables showing the results of the experiments it may be seen that in no case was the B.O.D. significant enough to affect the results of the tests.

Tests were conducted as described below to determine the approximate oxygen consumption of individual mayfly nymphs under laboratory conditions. Six sample bottles of 250 milliliter capacity each were cleaned, sterilized and then filled to the top with tap water. Samples one and two were then used to determine the dissolved oxygen content of the water prior to beginning the experiment. Samples three and four were stoppered without air and placed in incubation at 12 degrees centigrade. Samples five and six received ten mayfly nymphs (lengths

approximately 10 m/m each.). These samples were tightly stoppered and placed in incubation at 12 degrees centigrade also. At the end of four days (96 hours) each of the four samples was tested for dissolved oxygen content. The difference between the average value in samples three and four and that of one and two was presumably due to the B.O.D. of the tap water. The difference between the average value for samples five and six from that of one and two was probably due then to the B.O.D. of the water plus the oxygen consumption of 10 insects in the former samples.

To calculate the approximate daily consumption of oxygen by each nymph, the average value for samples three and four was subtracted from the average of one and two. This figure then was substracted from the average of the values of five and six, giving the total dissolved oxygen consumption of 10 nymphs for a four-day period. The daily consumption for each nymph then would be this value divided by 40 (10 x 4).

TABLE 1. DETERMINATION OF THE DAILY OXYGEN CONSUMPTION OF MAYFLY NYMPHS

Test 1

Sample	Initial Dissolved Oxygen p.p.m.	Average	Final Dissolved Oxygen p.p.m.	Average	
1	11.2	11.2			
2	11.2				
3			11.0	10.9	0.3*
4			10.8		
5			9.2	9.6	1.9**
6			9.4		

* Value of average of samples three and four subtracted from average of samples one and two.

** Value of average of samples five and six subtracted from average of samples one and two.

The daily consumption per individual mayfly nymphs would be:

$$\frac{1.9 - 0.3}{40} = .04 \text{ p.p.m.}$$

Test II

Sample	Initial Dissolved Oxygen p.p m.	Average	Final Dissolved Oxygen p.p.m.	Average	
1	11.2	11.2			
2	11.2				
3			11.0	10.95	.25*
4			10.9		
5			9.1	9.15	2.05**
6			9.2		

* Value of the average of samples three and four subtracted from the average of samples one and two.

** Value of the average of samples five and six subtracted from the average of samples one and two.

The daily consumption per individual nymph would be:

$$\frac{2.05 - .25}{40} = .045 \text{ p.p.m.}$$

The average daily consumption per individual would then be approximately .04 p.p.m. for both Test I and II. The significance that may be drawn from this experiment is that under the conditions in the laboratory and consequently in the testing containers, the oxygen consumption of the number of nymphs used in each test, by respirational activities would not be great enough to lower the dissolved oxygen content to any serious degree during any test.

Determination of Alkalinity

Alkalinity of natural waters is usually attributable to the presence of calcium and magnesium carbonates, bicarbonates and hydroxides. Van Horn (1946) states that although the toxic affect of alkalinity is relatively less than that of sulfides, mercaptans, and soaps, the determination of alkalinity is important in tests of this nature. Alkalinity values then were found for the test solutions to determine whether or not this was an important factor in the mortality of the test animals in the test solutions. Values over 200 p.p.m. of $CaCO_3$ and methyl orange alkalinity were considered as being significant as factors in mortality of insects.

Alkalinity is defined in terms of parts per million of calcium carbonate by methyl orange titrations. The actual procedures used in determinations were taken from Standard Methods for the Examination of Water and Sewage (1946).

EXPERIMENTS CONDUCTED WITH EPHEMEROPTERA

In these experiments the approximate minimum lethal concentration was arbitrarily selected to be the concentration of the chemical in solution that would rather consistently be lethal to more than 50 percent of the test animals in less than 48 hours, or that concentration that would be lethal to over 80 percent of the nymphs in less than 96 hours. In some cases it was found that even though a chemical solution would not be lethal to more than a small percent of the nymphs within 48 hours, the concentration was usually high enough to become lethal to more than 80 percent of the nymphs before 96 hours (See Tables 6 and 11). In most instances the former criterion was applicable in setting up the minimum lethal concentrations. Generally, although the results of the tests of a chemical of any one concentration were varied, the minimum lethal concentration (M.L.C.) of any chemical studied would be sufficiently toxic to cause about 100 percent mortality in a matter of 72 or 96 hours. At lower concentrations a certain percent of the test animals ordinarily remained alive beyond these periods.

The selection of the test concentrations at the beginning of each experiment was based somewhat upon the M.L.C. obtained by Haydu (1949) in experiments made on silver and chinook salmon. It was anticipated that the M.L.C.'s would be about the same for salmon and for the

insects used. In the case of hydrogen sulfide, the M.L.C. for silver salmon fingerlings was about 1.25 p.p.m. and for chinook salmon 1.0 p.p.m. With these figures as a guide, the first concentrations used in the series of tests involving Ephemeroptera nymphs and hydrogen sulfide was 1.0 p.p.m., 2.0 p.p.m., and 6.8 p.p m. Table 2 shows that 2.0 p.p.m. and 6.8 p.p.m. were lethal in a short time; 1.0 p.p.m. was not lethal. Accordingly, the next test concentrations selected were between 1.0 p.p.m. and 2.0 p.p.m. This method of elimination or "screening" was used until the minimum lethal concentration was established.

The testing containers used were 250 milliliter sample bottles. The use of these bottles simplified the work somewhat by making dissolved oxygen determinations at the end of a test more accurate and convenient. After the duration of a test, it was necessary only to add the prescribed Winkler reagents to the bottle, which had been filled to the top for the test, and proceed with the determination. Since nymphs once used for a test were discarded, they were not removed from the bottle for the determination of the dissolved oxygen. In case it was desired to make dissolved oxygen, pH and alkalinity tests on the contents of the same test container, 100 milliliters, a volume that may be used for dissolved oxygen determination, was carefully siphoned into a 100 milliliter volumetric flask. The pH and alkalinity determinations

were then made on the remaining 150 milliliters of solution.

In setting up the test, the test containers were filled (about 250 milliliters) with the test solutions; usually two or more containers with the same concentrations. Dissolved oxygen, pH and alkalinity determinations were made on one or two of the representative samples. The others were set aside to receive the test animals. In case of mayfly nymphs, five or ten individuals were placed into each test container. Each bottle was numbered with the sample number, the name of the pollutant and its concentration.

Observations were made on the tests at frequent intervals for a period of 48 to 96 hours. The length of each test depended somewhat upon the stage of completion of the experiment. Mortality and reactions of the nymphs was recorded at 24 hour intervals.

Only healthy nymphs were used as test animals. Their length varied from three to 10 millimeters. No appreciable difference was noted between the smaller and larger specimens as to susceptibility to the toxic qualities of a chemical. Control tests conducted simultaneously with the regular tests, indicated that virtually all nymphs would live "indefinitely" in tap waters if untreated by any unnatural chemical substance, under conditions identical to those the chemical tests were subjected.

Affect of Chemical Solutions on Test Animals

1. Hydrogen sulfide

Very low concentrations of hydrogen sulfide were fatal to mayfly nymphs. As shown by Table 2, concentrations of 1.50 p.p.m. were usually fatal to most of the insects within 48 hours. At concentrations of 1.40 p.p.m. most were still alive after 48 hours. However, in one test, 1.40 p.p;m. was lethal to all test animals in 24 hours. Variations of this type occur occasionally as may be seen by the Table 2 and the other tables. These variations generally occur in concentrations to either side and near the minimum lethal concentration. Very high or very low concentrations were practically always completely lethal or completely non-lethal, respectively. No attempt is made at this time to explain these differences of affect. Haydu was confronted with somewhat similar results in some of his work with salmon especially that with sodium sulfate and sodium thiosulfate.

2. Sodium sulfide

This chemical was lethal to more than 50 percent of the nymphs in 48 hours at a concentration of 170 p.p.m. In the lethal strengths, death was rapid for a large percent of the nymphs. In sodium carbonate testing, for example, as much as 24 hours passed before the toxic

properties began to affect the nymphs; death occured shortly thereafter however. Solutions of sodium sulfide are of high pH qualities. For solutions of 170 p.p.m., the pH was about 11.0 at the beginning of the tests. This value is probably near the maximum variance toward basicity, away from a neutral solution, that is tolerable by most aquatic forms. The pH may well have been adjusted to about neutral for more certainty as to the fact that the test animals were killed by the toxic properties of the chemical itself rather than by the resultant rise in pH.

3. Sodium hydroxide

Solutions of this chemical clouded in the test containers after the tests had been in progress for a day or more. At some sub-lethal concentrations the solution had a depressing affect on the nymphs. Their actions were definitely dull, however, they survived the testing and thus the solutions could not be considered lethal. With sodium hydroxide as with sodium sulfide, the pH of the solutions was high, however, probably still within ranges not detrimental to aquatic animals by virtue of the hydrogen ion concentrations alone. Table 4 shows that at times the alkalinity was relatively high and may have affected the test animals adversely.

4. Sodium carbonate

In many instances during the testing, sodium carbonate solutions were seemingly not toxic until a period of about 48 hours had elapsed. After this time the mortality among the test animals was high in certain concentrations. For example, as shown in Table 5, 500 p.p.m. in one test killed no nymphs in a test container holding 10 individuals during the first 24 hours of the test. Conversely, during the second 24 hour period, all 10 nymphs were killed.

The alkalinity of the M.L.C. was relatively high and may have been a factor in the toxicity of the solution.

5. Sodium sulfate

At concentrations above 7000 p.p.m. the nymphs in the test solutions would become decidedly slow in movement after about 48 hours, somewhat as in the case of the testing with sodium hydroxide. About 5500 parts per million concentrations were necessary to become lethal. Being a neutral salt, this chemical made little if any changes in the pH of any solutions. The lethal concentrations were rather high, 11000 p.p.m. and the results varied considerably. A solution of 5500 p.p.m. proved lethal to one group of nymphs in 96 hours while one solution of 7000 p.p.m. proved to be lethal to only 20 percent of the

nymphs in 96 hours. The concentration lethal to about 50 percent of the nymphs in 48 hours in most cases was 5500 p.p.m.; nearly all nymphs were dead at 72 or 96 hours. At concentrations under 5500 p.p.m. the nymphs that remained alive appeared to be unusually active, more so than those nymphs used in the control tests.

6. Sodium hydrosulfide

As with hydrogen sulfide solutions, the hydrosulfide solution was lethal in weak concentrations. It was noted that as the concentrations were increased that the percent of the test animals killed increased gradually up to a point where over 50 percent were killed in 48 hours. With some of the pollutants the M.L.C. was more distinctly set apart, as was the case with sodium carbonate and sodium sulfide. Two samples, eight p.p.m. and 10 p.p.m., prepared as usual and containing 10 nymphs each were tightly stoppered to determine the combined consumption of dissolved oxygen by the B.O.D. of the test solution and by the mayfly nymphs. The dissolved oxygen values at the beginning of the testing were 11.2 and 10.6 respectively. After 72 hours the dissolved oxygen content for the sample containing eight p.p.m. was 0.9 p.p.m.; for the 10 p.p.m. sample, 0.8 p.p.m. This indicates that the B.O.D. of sodium hydrosulfide solutions under the conditions of these tests may be relatively high. In the usual

biological assays involving this chemical, the containers were not stoppered, permitting the proper conditions apparently for maintainence of a safe oxygen level.

Interpretation of Results

The application of the results of this study as presented, toward the solution of the problems under consideration is hardly justifiable. It cannot be said with certainty at this point that any particular minimum lethal concentration of any pollutant as defined and determined herein, would be detrimental to aquatic insects such as mayfly and stonefly nymphs under natural conditions. Many factors not considered in this work would play a part in the biological balance of a natural environment. Much larger volumes of solution, continuous supplement of the oxygen supply, proper food supply, and freedom of movement are some of the factors that would be encountered by the nymphs in polluted waters of a "natural" environment. In all likelihood the M.L.C. of the various pollutants might be different from those M.L.C. found under laboratory conditions. In some cases higher concentrations may be necessary to become lethal; in other cases lower. These experiments do provide an indication as to what the actual situation may be in certain polluted areas.

Except in solutions of sodium hydroxide and sodium sulfate, the mayfly nymphs utilized are probably more resistant to the solutions than are chinook and silver salmon fingerlings under almost identical conditions.

TABLE 2 - THE EFFECT OF HYDROGEN SULFIDE SOLUTIONS ON CALLIBAETIS SP. NYMPHS

Concentration p.p.m.	pH Start	pH End	Dissolved Oxygen p.p.m. Start	Dissolved Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent Mortality in 24	48	72	96 hrs.	No. Animals
.85							0	0			10
1.00							0	10			10
1.00							10	10			10
1.00							0	0			10
1.25							0	10			10
1.25							60	80			10
1.30	7.5	7.0	12.0	11.4	37	34	0	0			10
1.36	7.5	7.5					0	20			10
1.36	7.5	7.5					0	0			10
1.36	7.5	7.5					0	0			10
1.40	7.0	6.6	11.0	10.8	38	36	20	30			10
1.40	7.0	7.0	11.0	10.6	38	36	20	30			10
1.40	7.5	7.5			38	36	100				10
1.40	7.5	7.5			38	36	0	20			10
1.40	7.5	7.5			38	36	0	30			10
1.40	7.5	7.5			38	36	0	0			10
1.50*	7.5	7.5			36	36	0	0			10
1.50	7.3	7.0	11.2	10.8	40	36	100				10
1.50	7.3	7.1	11.2	10.6	40	36	70	90			10
1.50	7.5	7.5					70	80			10
1.50	7.5	7.5					30	70			10
1.50	7.5	7.5					30	90			10
1.60							0	0			10
1.70	7.3	7.0	11.0		37	34	100				10
1.70	7.0	7.5					70	70	90		10
1.75	7.0	7.5					100				10
							100				10

TABLE 2 - THE EFFECT OF HYDROGEN SULFIDE SOLUTIONS ON CALLIBAETIS SP. NYMPHS (CONT)

Concentration p.p.m.	pH Start	pH End	Dissolved Oxygen p.p.m. Start	Dissolved Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent Mortality in 24 hrs.	48 hrs.	72 hrs.	96 hrs.	No. Animals
2.00	7.0	7.5					100				10
3.40	6.5	6.5					100				10
6.80	6.5	6.5					100				10
17.00	6.0	6.0					100				10
34.00	4.5	4.5					100				10

* Approximate minimum lethal concentration.

TABLE 3 - THE EFFECT OF SODIUM SULFIDE SOLUTIONS ON CALLIBAETIS SP. NYMPHS

Concentration p.p.m.	pH Start	pH End	Dissolved * Oxygen p.p.m. Start	Dissolved * Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent Mortality in 24	48	72	96 hrs.	No. Animals
25	9.0	8.0					0	0	0		10
75	11.0	8.0					0	0	0		10
125	10.5	8.0					0	20	20		10
125	10.5	8.0					0	20	20		10
140	10.5	8.0					0	0	0		10
140	10.5	8.0					0	0	0		10
150	10.5	8.5	9.0				0	0			10
150	10.5	8.5	9.0				100				10
150	10.5	8.5	9.1				0	60			10
150	10.5	8.5	9.1				0	0			10
155	10.5	8.5	9.1				60	100			10
155	10.5	8.5	9.1				0	0			10
155	10.5	8.5	9.0				0	0			10
160	10.5	9.0	9.0				0	0			10
160	10.5	9.0	9.1				0	0			10
160	10.5	9.0	9.1				0	80			10
170**	11.0	9.0	9.1		58	68	60	80			10
170	11.0	9.0	8.9		61	65	0	30			10
170	11.0	9.0	8.9		61	65	20	30			10
170	11.0	9.0	9.1		55	68	40	80			10
170	11.0	9.0	9.1		55	68	50	90			10
170	11.0	9.0	9.0		53	70	10	80			10
170	11.0	9.0	9.1		55	68	30	90			10
170	11.0	9.0	9.1		55	68	50	90			10
170	11.0	8.7					10	40	60		10
170	11.0	9.0					10	10	10		10
170	10.5	9.3					20	40	80		10

TABLE 3 - THE EFFECT OF SODIUM SULFIDE SOLUTIONS ON CALLIBAETIS SP. NYMPHS (CONT.)

Concentration p.p.m.	pH Start	pH End	Dissolved* Oxygen p.p.m. Start	Dissolved* Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent mortality in 24	48	72	96 hrs.	No. Animals
170	10.5	9.4					10	60	90		10
175	11.0	9.5	9.0		53	70	40	100			10
180	11.0	9.5	9.0		53	70	30	90			10
180	11.0	9.5	9.0		53	70	10	70			10

* Determination of the dissolved oxygen content of the tap water before test solution was prepared.

** Approximate minimum lethal concentration.

TABLE - 4 - THE EFFECT OF SODIUM HYDROXIDE SOLUTIONS ON CALLIBAETIS SP. NYMPHS

Concentration p.p.m.	pH Start	pH End	Dissolved Oxygen p.p.m. Start	Dissolved Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent mortality in 24	48	72	96 hrs.	No. Animals
10											10
25	10.0	9.6	11.2	10.5	90	120	0	0			10
25	10.0						0	0			10
25	10.7				382		0	0	10	20	10
25	10.7				382		0	0	20	30	10
27*	10.8	10.7			402		100				10
27	10.8	10.7			402		100				10
29	10.8	10.7			422		100				10
29	10.8	10.7			422		100				10
30	10.8	10.7	11.2	9.0	414		60	100			10
30	10.8	10.7	11.2	9.0	414		100				10
35	10.8	10.7	11.2	9.0	486	532	100				10
35	10.8	10.7	11.2	9.0	486	532	100				10
40	10.9	10.8	11.1	8.9	542		100				10
40	10.9	10.8	11.1	8.9	542		100				10
40	9.9	9.2	11.0	10.9	85	80	80	100			10
40	9.9	9.0	11.0	10.5	85	82	60	100			10
60	10.0	9.7	11.7	11.2	86	118	100				10
60	10.0	9.8	11.7	11.2	86		100				10
70							100				10
70							100				10
100	10.5	9.8	11.0	10.8	91	126	100				10
100							100				10
200							30	90			10
300							100				10
400							100				10
500							100				10
1000							100				10

* Approximate minimum lethal concentration.

TABLE 5 - THE EFFECT OF SODIUM CARBONATE SOLUTIONS ON CALLIBAETIS SP. NYMPHS

Concentration p.p.m.	pH Start	pH End	Dissolved Oxygen p.p.m. Start	Dissolved Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent mortality in 24	48	72	96 hrs.	No. Animals
50	9.0	8.5	11.0	10.0	80	85	0	0			10
100	9.2	8.4	10.9	9.9	120	118	0	0			10
200	9.2	8.4	10.9	9.8			0	0			10
250	9.3	8.3	11.0	9.9			0	0			10
300	9.7	8.9	10.8	10.0			0	0			10
400	9.7	9.0	10.8	10.1			0	0			10
400	9.3	9.0	11.2	9.5			0	20			10
450	9.4	9.1	11.2	10.0			0	30			10
450							0	20	20	40	10
450							0	0	0	0	10
470	10.2	8.7	11.1		510	800	0	0	0	0	10
470	10.2	8.7	11.1		510	800	0	0	0	0	10
480	10.3	8.7	11.0		526	840	0	10	10	10	10
480	10.2	8.8	11.2	10.8	526	840	0	10	10	10	10
500*	10.2	8.8	11.2	10.7	534	880	0	0	0	0	10
500							70				10
500							40				10
500	9.8	9.2	10.9	10.1			0	20			10
500	9.5	8.9	11.3	10.1			0	80			10
500	9.5	8.9	10.0				0	100			10
500							20	20	40	60	10
525	9.5	8.7	11.0				20	100			10
525							60				10
525							80				10
550	9.5	8.9	11.0	10.2			20	100			10
550	9.3	9.0	10.0				80	100			10
600	9.6	8.9	11.0	10.8				40			10
600	9.5	9.0	11.0	10.2			40	100			10
800	9.6	8.8	10.4	10.0				60			10

TABLE 5 - THE EFFECT OF SODIUM CARBONATE SOLUTIONS ON CALLIBAETIS SP. NYMPHS (CONT.)

Concentration p.p.m.	pH		Dissolved Oxygen p.p.m.		Alkalinity		Percent mortality in				No. Animals
	Start	End	Start	End	Start	End	24	48	72	96hrs.	
1000	9.8	9.0	10.4	9.5			100				10

* Approximate minimum lethal concentration.

TABLE 6 - THE EFFECTS OF SODIUM SULFATE SOLUTIONS ON CALLIBAETIS SP. NYMPHS

Concentration p.p.m.	pH Start	pH End	Oxygen p.p.m. Dissolved Start	Oxygen p.p.m. Dissolved End	Alkalinity Start	Alkalinity End	Percent mortality in 24	48	72	96 hrs.	No. Animals
2000							0	0	0		10
3000							0	0	0		10
4000							0	0	0		10
5000	7.5	7.5	11.4	10.0	56	56	80	0	0		10
5000							0	0	0		10
5000							20	30	50		10
5000							0	0	0		10
5500*							0	20	40	100	10
5500							0	20	40	100	10
6500							0	60	60	100	10
7000**							0	60	80	80	10
7000							20	20	40	60	10
7000	7.5	7.5	11.0	8.9	55	54	0	0	40	100	10
7000	7.5	7.5	11.0	8.8	55	54	0	0	0	20	10
7000	7.5	7.5	11.4	10.0	55	54	0	20	40		10
8000											10
8000											10
9000	7.5	7.4	11.0	8.8	58	55	0	20	60	80	10
9000	7.5	7.4	11.1	8.9	58	55	0	40	60	100	10
10000							20	40	100		10
10000							0	80	100		10
12000	7.4	7.5	11.0	10.8	20	64	0	20	40	100	10
12000	7.4	7.5	11.0	10.8	20	64	0	40	60	100	10
12500	7.5	7.5	11.9	10.7	50	46	40	80			10
12500	7.5	7.5	11.9	10.7	50	46	40	80			10
13000	7.4	7.5	11.2	10.8	22	69	0	20	20	80	10
13000	7.4	7.5	11.2	10.8	22	69	40	60	60	100	10
15000	7.5	7.3	11.0	9.5	50	50	40	100			10
20000	7.5	7.3	11.2	9.3	65	60	100				10

TABLE 6 - THE EFFECTS OF SODIUM SULFATE SOLUTIONS ON CALLIBAETIS SP. NYMPHS (CONT.)

Concentration p.p.m.	pH Start	pH End	Dissolved Oxygen p.p.m. Start	Dissolved Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent mortality in 24	48	72	96 hrs.	no. Animals
30000	7.5	7.4	10.9	9.0			100				10

Approximate

* Minimum lethal concentration

** At a concentration of 7000 p.p.m. and above all nymphs became very sluggish after 48 hours even though remaining alive.

TABLE 7 - THE EFFECT OF SODIUM HYDROSULFIDE SOLUTIONS ON CALLIBAETIS SP. NYMPHS

Concentration p.p.m.	pH Start	pH End	Dissolved Oxygen p.p.m. Start	Dissolved Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent mortality in 24	48	72	96 hrs.	No. Animals
1	7.3	6.8	9.9	9.1	45	50	0	0			10
1	7.3	6.9	9.9	9.2	45	50	20	20			10
2	7.5	7.0	10.0	9.1	40	46	0	0			10
2	7.5	7.0	10.0	9.1	40	45	20	20			10
3							20	20			10
3							0	0			10
4							40	40			10
4							40	60			10
5	7.5	7.4	10.4	9.9			0	20			10
5	7.4	7.3	10.4	10.1			0	0	0	0	10
5							20	20	60	60	10
5							20	20	20	20	10
5	7.5		11.4	10.7	30	31	0	0	0	0	10
6	7.5		11.4	10.3	35	35	60	60	60		10
6	7.5	6.7	11.4	5.4	35	35	0	60	60		10
6							20	40	60		10
6							20	60	60		10
6	7.5	7.4	10.9	8.6	30	38	0	0	0	20	10
6	7.5	7.4	10.9	8.6	30	38	0	0	10	10	10
7	7.5		11.0	10.0	35	36	0	0	10	10	10
7	7.5	6.6	11.0	10.8	35	35	20	60	80		10
7							20	40	40		10
7							40	40	40	40	10
7	7.5	7.4	10.9	8.6	30	38	20	20	40	40	10
7	7.5	7.4	10.9	8.6	30	38	0	10	10	10	10
8*	7.5	7.4	10.9	8.5	30	39	0	20	20	40	10
8							0	20	20	20	10
8	7.5	6.5	11.2	0.9	36	36	40	60	80		10
							60	80	100		10

TABLE -7-THE EFFECT OF SODIUM HYDROSULFIDE SOLUTIONS ON CALLIBAETIS SP. NYMPHS (CONT.)

Concentration p.p.m.	pH		Dissolved Oxygen p.p.m.		Alkalinity		Percent mortality in				No. Animals
	Start	End	Start	End	Start	End	24	48	72	96 hrs.	
8							40	80	100		10
9							40	70	90		10
9							80	100			10
10							80	100			10
20	7.5	7.0	10.6	0.8	36	37	100				10

* Approximate minimum lethal concentration.

EXPERIMENTS CONDUCTED WITH PLECOPTERA

The test with stonefly nymphs were carried out in much the same manner as were the tests made with mayfly nymphs. However, since the Plecoptera nymphs were generally less hardy than the mayfly larvae, modifications of the testing procedures were made. Wide-mouthed, four by one and one-half inch finger bowls of 300 milliliter capacity were used as testing containers. These containers provided more surface area of the solutions which aided in keeping the dissolved oxygen content at a higher level and facilitated handling of the nymphs. In each container a rubber stopper was provided to which the nymphs could cling. During a test they would seldom leave the stoppers and would remain attached tightly to them even though they were lifted out of the solution and transferred elsewhere.

The nymphs used in testing were from 12 to 25 millimeters in length and were the species Acroneuria pacifica. This species belongs to the family Perlidae. Three individuals were placed into 200 milliliters of testing solution for most of the tests. The chemicals used as pollutants in the testing were sodium sulfide, sodium hydroxide, sodium sulfate, and sodium carbonate. Ordinarily a large volume of solution was prepared for each concentration that was desired for testing. Prior to the tests

the quality determinations were made from the large sample. Determinations that were necessary to make at the end of any set of tests were made from the actual test solutions.

As with Ephermeroptera testing, "control" tests were carried on concurrently with all significant chemical testing. These "control" solutions were samples of the tap water, without any unnatural concentrations of chemicals, that received test animals in the same manner and under the same conditions as the regular test samples. By running "controls" it was possible to compare the results of a test with pollutants with the results of tests with untreated water. It was assumed that if, under any one set of conditions, the test animals in the untreated water survived while the animals in a treated sample perished within the testing period that the deaths were due to some unnatural cause and not by factors present in the "natural" untreated water.

In the case of Plecoptera because of their more delicate nature it was found that frequently nymphs would die in a day or so after being placed in an untreated solution in a test container. For example, in a "control", three apparently healthy nymphs were placed. In eight or 10 hours it was a common occurence for one to be dead while the other two lived on for a matter of weeks. It is assumed that the nymph that died was not as easily adaptable to test conditions as the others or it may

have been injured in some way during handling. However, since a significant number of nymphs were of such unreliable nature under these circumstances the minimum lethal concentrations chosen were those concentrations at which all nymphs died during the testing up to 96 hours. Thus a concentration that soon proved lethal to one or two nymphs out of three, but permitted the remainder of the number to live on for four or more days, was not considered a lethal concentration.

Effect of Chemical solutions on Test Animals

Only little may be added to the remarks regarding the effects of the pollutants on the mayfly nymphs in relation to the effects on stonefly nymphs. In the case of sodium sulfide solutions, stonefly nymphs did not show increased activity in any of the various concentrations of sodium sulfate as did the mayfly nymphs. Ordinarily, as shown by comparing the tables on mayfly nymph testing and stonefly nymph testing, the dissolved oxygen consumption was apparently greater for three stonefly nymphs than for 10 mayfly nymphs. The minimum lethal concentration of each compound is indicated on each table showing the results of the experiments.

1. Sodium Sulfide

About 55 p.p.m. was the approximate M.L.C. in the

case of sodium sulfide on stonefly nymphs. With mayfly nymphs the M.L.C. was about 170 p.p.m. In this instance stonefly nymphs showed considerably less resistance to the chemical than did the mayfly nymphs. Mortality occurred rapidly, comparing somewhat with the rapid mortality among mayfly young using hydrogen sulfide as the test solution. In either case the hydrogen sulfide was the toxic agent and was quickly effective.

2. Sodium Hydroxide

Stonefly nymphs again showed slightly less resistance to solutions of this chemical than did mayfly nymphs. Considerable variations were noted in the speed of action on the nymphs. At 100 p.p.m. for example, only 60 percent of the test animals were killed within 24 hours during one test even though this concentration is far above the approximate M.L.C. After death the nymphs quickly lost their body color and turned to a "blistered", reddish shade.

3. Sodium Carbonate

The concentration necessary to become lethal to the stonefly nymphs was almost twice that required to kill mayfly nymphs. The high alkalinity of these solutions may be responsible in part for the mortality that occurred. In this case the M.L.C. is given only roughly.

Probably additional tests should be carried out to narrow down the limits shown in Table 10.

4. Sodium Sulfate

Solutions of this chemical were often slow in affecting the test animals. Even at the M.L.C. (24,000 p.p.m.), mortality did not occur until the testing had progressed about 48 hours. At higher concentrations, the same results were observed. The concentrations necessary to kill the stonefly nymphs in accordance with the selected definition of M.L.C. was over three times that required to produce a M.L.C. for mayfly nymphs. No concentrations used appeared to increase the activity of the nymphs as occured in the testing involving the immature mayflies.

INTERPRETATION OF RESULTS

The results of this phase of the project are probably no more or less significant than those discussed for Ephemeroptera experiments. That which was said in reference to the mayfly nymph testing may well be said of these findings

In some cases the stonefly nymphs proved to be more resistant to the toxicity of the chemicals than did mayfly nymphs (Table 12). It has long been supposed that stoneflies provide an "index" to stagnation and pollution; not to be found ordinarily under these conditions. According to the observations made it would seem that Acroneuria pacifica nymphs are as much or more an "index" than are Callibaetis sp. nymphs. These conclusions do not prove the point; rather merely indicate the possibility of a deviation from the regularly accepted idea.

There is little doubt that Acroneuria nymphs would be resistant to even greater concentrations of the various chemicals if experiments could be carried out under conditions more nearly that of the natural habitat. Acroneuria probably possess a "current demand" and unless current in combination with aeration, could be provide, the test results would not be as accurate as possible.

TABLE 8 - THE EFFECTS OF SODIUM SULFIDE SOLUTIONS ON ACRONEURIA PACIFICA NYMPHS

Concentration p.p.m.	pH Start	pH End	Dissolved * Oxygen p.p.m. Start	Dissolved * Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent Mortality in 24	48	72	96 hrs.	No. Animals
10							0	0			3
50							0	0			3
50							0	0			3
50	10.4	10.9			68	72	30	60	60		3
55**	10.5	10.9			72	80	100				3
55	10.5	10.9			72	80	60	100			3
55	10.5	10.9			72	80	60	100			3
60		9.0				86	100				3
70		9.1				96	100				3
80		9.1				106	100				3
90		9.2				112	100				3
100							100				3
250	10.9	10.0	11.1		326	246	100				3
300	11.0	11.0	11.1		388	388	100				3
500	11.0	11.0	11.1		628	628	100				3
1000	11.0	11.0	11.1		1236	1236	100				3

* Determination of the dissolved oxygen content of tap water before test solutions were prepared.

** Approximate minimum lethal concentration.

TABLE 9 - THE EFFECTS OF SODIUM HYDROXIDE SOLUTIONS ON ACRONEURIA PACIFICA NYMPHS.

Concentration p.p.m.	pH Start	pH End	Dissolved Oxygen p.p.m. Start	Dissolved Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent mortality in 24 hrs.	48 hrs.	72 hrs.	96 hrs.	No. Animals
10	9.3		8.3				0	0	0	30	3
10							0	0	0	0	3
10							0	0	0	0	3
10							0	0	30		3
12							0	0	30		3
14							0	0	30		3
14							60	60	60		3
14							30	30			3
14							60	60			3
15*	10.0	9.8	11.6	11.3			30	60			3
15	10.0	10.0	11.0	10.8			30	30			3
15							100				3
15							0	60	100		3
15							30	90			3
20	10.0	9.9	11.0	10.8	90	120	60	60	100		3
20							60				3
30	9.9	9.8	11.1	10.8	88	100	100				3
50	10.0	9.9					30	100			3
50	10.0	10.0					100				3
100							60	100			3

* Approximate minimum lethal concentration.

TABLE 10 - THE EFFECT OF SODIUM CARBONATE SOLUTIONS ON ACRONEURIA PACIFICA NYMPHS.

Concentration p.p.m.	pH Start	pH End	Dissolved Oxygen p.p.m. Start	Dissolved Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent mortality in 24 hrs.	Percent mortality in 48 hrs.	Percent mortality in 72 hrs.	Percent mortality in 96 hrs.	No. Animals
100							0	0	30		3
300							0	0	0	0	3
400							0	0	0	0	3
500							0	0	0	0	3
500							0	30	30	30	3
500	10.4	8.8			532	790	0	0	0		3
600	10.4	8.8			532	798	0	0	30	60	3
700	10.4	8.8					0	0	0	0	3
800	10.3	8.7	10.8	8.9			0	0	0		3
900*	10.3	10.0			630	930	0	60	100		3
900			11.0	9.8	630	960	30	100			3
950	10.4	10.0					100				3
1000	10.8	10.0			982	1200	60	100			3
1000	10.5	10.0	11.0	8.2		1160	60	60	100		3
1500	10.8	10.0				1810	100				3
1500	10.5	10.0					100				3
2000	10.9	10.0				2220	100				3

* Approximate minimum lethal concentration.

TABLE 11 - THE EFFECT OF SODIUM SULFATE SOLUTIONS ON ACRONEURIA PACIFICA NYMPHS.

Concentration p.p.m.	pH Start	pH End	Dissolved Oxygen p.p.m. Start	Dissolved Oxygen p.p.m. End	Alkalinity Start	Alkalinity End	Percent mortality in 24	48	72	96 hrs.	No. Animals
1000							0	0	0	0	3
10000							0	0	0	0	3
15000							0	0	0		3
20000							0	0	0	0	3
20000							30	60	60	60	3
20000							0	0	30		3
22000	7.5	7.4	11.0	8.0	20	40	0	0	30		3
22000	7.5	7.4	11.0	8.0	20	40	0	0	30		3
24000*	7.5	7.4	11.0	7.6	20	42	0	100			3
24000	7.5	7.3	11.0	7.6	20	42	0	100			3
25000	7.4	7.2	11.2	5.6	22	42	0	30	100		3
25000	7.4	7.2	11.2	5.6	22	42	0	30	100		3
25000							0	30	30	60	3
25000							60	60	60		3
25000							0	3	100		3
25000							0	100			3
30000	7.4	7.3	11.2	5.6	26	42	0	100			3
30000	7.4	7.3	11.2	5.6	26	42	100				3
30000							0	60	100		3
30000							30	60	100		3
30000							100				3
35000							100				3
35000							100				3

* Approximate minimum lethal concentration.

CONCLUSIONS

It may be stated, generally, that with the exceptions noted and under the conditions cited, Ephemeroptera nymphs of the genus Callibaetis used and Plecoptera nymphs of the species Acroneuria pacifica are no less resistant to solutions of some of the common pulp and paper mill waste products components than are chinook and silver salmon fingerlings.

Solutions of sodium carbonate and sodium sulfate are less toxic to the stonefly nymphs than to mayfly nymphs. Sodium hydroxide in solution is roughly equally toxic to either species of nymphs while sodium sulfide is more highly toxic to stonefly nymphs than to mayfly nymphs.

The following table will serve to illustrate the above conclusions:

TABLE 12-THE RELATIONSHIP BETWEEN SALMON FINGERLINGS, STONEFLY AND MAYFLY NYMPHS IN RESPECT TO THEIR RESISTANCE TO TOXIC CHEMICALS

Toxic Compound	Na_2S ppm	Na_2CO_3 ppm	NaOH ppm	H_2S ppm	Na_2SO_4 ppm	NaHS ppm
Chinook Salmon	3.5*	68	50	1.00	12,500	3.3
Silver Salmon	3.0	75	20	1.25	16,500	3.5
Mayfly Nymphs	170.0	500	27	1.50	5,500	8.0
Stonefly Nymphs	55.0	900	15		24,000	

* Figures show the approximate minimum lethal concentrations.

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