AN ABSTRACT OF THE THESIS OF

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Artificial streams with continually renewed river water flowing over natural bottom materials in wooden troughs and often circulated by means of pumps to increase velocities were used to study experimentally the effects of pulp mill wastes on stream ecology. Representative stream animals were subjected in these streams, usually for two weeks or one month, to different concentrations of sulfite process pulp mill waste liquor, which was added constantly to the influent water; some were held likewise in unpolluted water with dissolved oxygen concentrations either normal or reduced by means of nitrogen. The long-term resistance of the organisms to harmful effects of the wastes, including reduction of dissolved oxygen alone, thus was evaluated. The utility of artificial streams in the identification of specific causes of death of animals in variously polluted waters was explored, and also their utility in the development of "toxicity bioassay application factors" intended for facilitating regulation of discharges of toxic wastes

of predetermined acute toxicity. The principal test animals were stonefly naiads of the genus <u>Acroneuria</u>, but other invertebrates and fish also were tested.

Both sulfite and kraft wastes produced striking changes in the artificial streams. A "slime" composed largely of <u>Sphaerotilus natans</u> sometimes completely blanketed bottom materials. Under the slime blanket, dissolved oxygen was greatly reduced. The variety and numbers of algae were much reduced in the presence of either waste. Perhaps because of the marked toxicity of kraft waste, neither chironomids nor cladocerans, which sometimes became numerous in streams receiving sulfite waste, were ever abundant in streams receiving kraft waste. Tested concentrations of kraft waste less than one-tenth the 24-hour median tolerance limit for young guppies (fish) at 20°C. did not increase the mortality rates of stonefly naiads nor cause much slime growth, whereas greater concentrations did.

Increased mortality of insects in the presence of kraft waste apparently was due largely to toxicity of the waste, but other factors such as reduced dissolved oxygen may have been partly responsible. Increased mortality in sulfite waste experiments was due largely to reduced dissolved oxygen, but probably in part also to some other factors, such as toxicity of the waste or slime growth on the insects, which may interfere with respiration. These conclusions are based on observed relationships between mortality rates of the insects and dissolved oxygen and waste concentrations in streams receiving the wastes and supplied with natural water or water that had been treated (oxygenated) with oxygen gas. They are based also on comparisons of curves relating mortality rates to the lowest recorded oxygen concentrations in these polluted streams and in streams with unpolluted water whose oxygen content had been suitably adjusted.

The low-oxygen tolerance of Acroneuria naiads at temperatures ranging from 2.2 to 24°C. and different water velocities was evaluated additionally through experiments wherein the animals were held (in cages) in tubular glass chambers with circulated water or in jars of standing water. The tolerance increased with increasing water velocity. The 24-hour median tolerance limits of reduced dissolved oxygen determined for A. californica naiads ranged from 0.6 mg/l at 2.2°C. and 0.13 feet per second velocity to 3.7 mg/l at 24°C. in standing water. Relationships between ventilatory movements, oxygen concentration, and water temperature were determined. Oxygen consumption rates at 20°C. in standing water with widely varying oxygen concentrations were determined; they

proved dependent on concentration even at levels near air-saturation.

The usefulness of artificial streams in studies of stream ecology and their limitations are fully discussed, and shortcomings of the reported experiments and some possible improvements are considered.

EFFECTS OF POLLUTIONAL CONDITIONS
ON STREAM ORGANISMS WITH ESPECIAL
EMPHASIS ON STONEFLY NAIADS

by

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONT.)

LIST OF FIGURES

Page

Page

Page

Page

LIST OF TABLES

EFFECTS OF POLLUTIONAL CONDITIONS ON STREAM ORGANISMS WITH ESPECIAL EMPHASIS ON STONEFLY NAIADS

INTRODUCTION

Changes in the physical and chemical features of streams and in their plant and animal communities take place when waste materials are discharged into stream waters. The great complexity of the changes that can occur under circumstances of pollution of aquatic environments has been indicated by a number of writers, including Richardson (38, p. 387-475), David (10, p. 132-169), Gaufin and Tarzwell (22, p. 906-923), and Hirsch (27, p. 500-553). However, the ways in which many wastes cause the observed effects on communities are little understood. Also, the responses of individual aquatic organisms to many environmental changes are not well known. The total response of an organism to what it faces where it lives has been termed the "biapocrisis" of that organism by Huntsman (29, p. 30). While the term biapocrisis has apparently not been widely adopted by biologists, Huntsman's examples in his explanation of its meaning clearly indicate the complexity of the response and of the interactions between various physical, chemical, and biological factors that can occur and that lead to the response. Only through a knowledge of the changes which take place in streams receiving wastes and the biapocrises of the organisms involved will it be possible to understand and predict changes in the aquatic communities. While the task of attaining an adequate understanding of these changes and their effects is difficult, the maintenance or improvement of valuable resources may depend on its completion.

Domestic sewage and other generally non-toxic organic wastes may not only produce conditions of dissolved oxygen deficiency, but may also produce growths of bacterial slime on bottom materials and on aquatic insects. Pulp mill waste liquors may be toxic as well as oxygen-depleting and slime-producing.

Many organisms may disappear or decline in numbers in streams receiving pulp mill waste liquors. Dymond and Delaporte (13) reported decreases in the numbers of many bottom organisms accompanying the deposition of wood fibers and the decline in the concentration of dissolved oxygen below a kraft process pulp mill outfall in the Spanish River in Canada. Biglane and Lafleur (4 p. 4, 5) reported the elimination of mayflies and molluscs from long stretches of rivers receiving kraft waste in Louisiana. Whitney and Spindler (52, p. 153) reported damage to bottom organisms and fish, and the formation of extensive growths of the sheathed bacterium, Sphaerotilus, in a Montana stream below a kraft mill outfall. When the numbers of invertebrate fish food organisms are altered in a section of stream receiving pulp mill wastes, is this due to toxicity, or to reduction of dissolved oxygen, or to the physical blanketing of the stream bottom with bacterial slime thriving on nutrients in the waste? What dilutions of various kinds of waste materials will be harmless to important stream organisms? How can laboratory experiments be best designed and their results applied in seeking solutions to problems in the ecology of natural streams? The discussion of these questions by Warren and Doudoroff (51, p. 211A-216A) is particularly pertinent to this thesis, the considerations they advance in connection with pulp mill waste control problems having led directly to the study reported herein. These writers point out that, while acute toxicity bioassays (such as those described by Doudoroff, et al., 12, p. 1380-1397) are useful in determining the concentrations of waste materials which are not lethal in a short time to the organisms being tested in a particular natural water, they do not directly measure or indicate the concentrations of wastes which would be harmless to these organisms over a long period. Conditions barely suitable for the survival of test animals for a short time in an aquarium are not likely to be compatible with unimpaired productivity of populations of these animals in a natural stream. The problem of how to apply the results of short-term toxicity bioassays to the control of waste discharges for the protection of aquatic resources thus presents itself.

It is suggested that the use of "artificial streams" will facilitate the study of the long-term tolerance of some organisms to waste materials and also the productivity of these organisms when they are subjected to some of the conditions found in clean and polluted natural streams, but with considerable control over many of the conditions. The results of short-term acute toxicity bioassays and of parallel, more prolonged experiments with artificial streams could provide the basis for the development of suitable formulas or "application factors," which after verification through studies on natural streams, could be used in deriving, from the results of routinely performed bioassays, reasonably reliable estimates of maximum biologically safe or harmless waste concentrations.

While artificial streams are not known to have been employed for the purposes outlined above before the present investigation, several successful attempts have been made to study some aspects of the ecology of streams by using simplified, artificially prepared habitats. Streeter (44, p. 1343-1346) who may have been the first to report the use of an artificial channel for such studies, used a metal channel 2 inches wide, 6 inches deep, and 4320 feet long to study the rate of purification of domestic sewage. A dilution of sewage in river water flowed constantly at a rate of 0.75 gallons per minute through the channel. Wu (56, p. 543-599) used V-shaped wooden troughs, whose slopes could be adjusted for the attainment of the desired water velocities, to study the oxygen and water current requirements of *Simulium* larvae in unpolluted water. She found that the larvae lived for prolonged periods at low concentrations of dissolved oxygen in the presence of a current but would quickly succumb in the absence of a current at the same or even higher oxygen concentrations. In standing water near air-saturation with oxygen, the larvae could live for long periods, indicating that the importance of current in this connection is in making available sufficient oxygen for the needs of the larvae. Wuhrmann (57, p. 212-220) used a system of sloping, parallel, concrete channels containing gravel and fed with river water containing sewage, for studies of the growth of "sewage fungus" at different sewage dilutions and flow velocities. The general objective of Wuhrmann's work was to determine the relationships between water chemistry and biological communities in water receiving sewage. Because of the inflexibility of the apparatus, the importance of current velocity as a factor influencing community development could not be studied. Zimmermann (58, p. 1-81), using channels which could be adjustably inclined for the production of different water velocities, was able to study the importance of current. In these latter channels, which contained gravel 30 to 40 mm. in diameter and received mixtures of groundwater and domestic sewage, he showed that velocity is no less important, or even more important, than the chemistry of waters receiving domestic sewage in determining the composition of the plant and animal communities therein.

Ambuhl (2, p. 133-264 and 3, p. 390-395) employed glass channels 6 cm. wide, 6 cm. deep, and 60 cm. long, containing model stones made of Plaster of Paris, to study the physical characteristics of water flow over stones and the effect of water velocity on the distribution of aquatic insects. Ambuhl's findings are discussed in more detail later, but generally he showed that current velocity is of major importance in determining the distribution of animals in a stream.

The significance of many ecological factors can be determined or estimated only by isolating or nearly isolating their effects. This can usually be accomplished only under highly controlled experimental conditions in specialized apparatus. The experiments with such apparatus are designed so that there can be only one variable, or very few variables of consequence. While artificial streams, containing various stream bottom materials and representing relatively complex environments, may be used effectively for some of this work, they do not lend themselves very well to all kinds of studies. Other apparatus often is more suitable.

Considerable work in which simple apparatus was employed has been done on the respiratory physiology of aquatic insect naiads and larvae. Heistand (25, p. 246-270) and Morgan and O'Neil (33, p. 361-379), for example, used small bottles for determinations of the oxygen consumption of caddisfly larvae in standing water. Wingfield (55, p. 363-373) determined the oxygen consumption of mayfly naiads in stirred water in bottles, and Philipson (36, p. 547-564) determined lethal oxygen concentrations for caddisfly larvae in stirred and unstirred water in bottles.

Ambuhl (2, p. 131-264 and 3, p. 390-395) employed bottles and "flow pipes" (tubes) in studying the effects of more or less isolated ecological factors, as well as his artificial channels, which were used for broader ecological studies. Ambuhl determined, among other things, the oxygen consumption rates of several species of stream insect naiads and larvae at different oxygen concentrations and water velocities at one temperature level (18-19°C.). His findings showed that oxygen uptake and lethal oxygen levels were generally dependent on oxygen concentration and water velocity in these animals.

This thesis reports preliminary experiments undertaken in an attempt to explore the usefulness of artificial streams for the study of the effects of pulp mill wastes on stream organisms, for the identification of individual factors (e.g., toxicity, reduced dissolved oxygen, etc.) which are responsible for observed effects on the organisms of the presence of such wastes, and for the development of simple empirical formulas ("application factors") for the practical application of routine toxicity bioassay results in the control of toxic waste discharges.

The stream experiments were conducted, using a wide variety of organisms, in wooden troughs in which either "pool" or "riffle" habitats were created. The use of such streams made possible the maintenance of rather constant conditions of waste liquor concentration, either from the standpoint of acute toxicity to reference animals, or from the standpoint of the total solids content. Also, the velocity of the water and the type of bottom materials were fairly well controlled. The main physical and chemical variables which were largely uncontrolled were the concentration of dissolved oxygen and water temperature.

It has been known for some time (Ebeling, 14, p. 192-200; Cole, 9, p. 280-302) that kraft process pulp mill wastes contain components toxic to fishes. Van Horn, Anderson, and Katz (49, p. 55-63) determined the levels of several components that are toxic to mayfly naiads and *Chironomus* larvae. The oxygen-depleting effects of sulfite waste liquor in natural waters are well-known (see for example, Tully, 47, p. 93-96). The artificial stream experiments made possible some evaluation of the effects on stream organisms both of toxic materials and of reduced oxygen concentrations that may occur in waters receiving kraft and sulfite process pulp mill wastes. Besides apparent toxicity to animals and oxygen depletion, bacterial "slime" production and other biological effects of the wastes were observed, and their influence on benthic animals was considered.

Because of the outstanding importance of dissolved oxygen depletion in both natural or artificial streams receiving pollutional materials such as pulp mill wastes, studies were also made of the tolerance of some aquatic insects to low oxygen concentrations in unpolluted water, and of other related aspects of their respiratory physiology. The determinations of low oxygen tolerance (over a wide range of water temperatures and velocities)and the related observations were made either in unpolluted artificial streams, in jars with standing water, or in tubes with flowing water. Only stonefly (Plecoptera) naiads were tested in the jar and tube experiments. Many of the results of this work parallel the results obtained by other workers for other species of aquatic insects.

The results of the artificial stream, the jar, and the tube experiments contribute to the base for the understanding of problems in the ecology of both polluted and unpolluted streams. The results of the polluted stream investigations are preliminary, but do provide some quantitative information and indicate further some of the value of artificial channels for such studies. Suggestions are made of ways in which artificial streams and studies on them could be improved for obtaining more meaningful results. The results of the jar and tube experiments are more nearly definitive and may have immediate application in the elucidation of the significance of some ecological factors.

APPARATUS, MATERIALS, AND METHODS FOR THE ARTIFICIAL STREAM EXPERIMENTS

Wooden troughs measuring 10 feet long, 10 inches wide, and 8 inches deep inside, and containing stream bottom materials and river water, were used in two different arrangements as artificial streams. For most of the stream "pool" experiments, four troughs were used, each receiving about 1,500 ml. per minute of river water directly from the water distribution system. These troughs were arranged in two pairs, each pair being illuminated continually by a 4-tube, 48-inch fluorescent light placed directly above. Each trough was divided transversely into two sections by a screen placed near the middle. Invertebrates were placed in the first section where water and waste liquors were introduced, while fish were held in the after section. With this arrangement the only appreciable current in the artificial stream pools was in the immediate vicinity of the water input tubes, each of which was constructed so as to produce a horizontal jet of water just beneath the water surface. Pulp mill waste liquor was mixed with the incoming water before it entered a trough.

In a second arrangement, two troughs, one placed above the other as shown in Figures 1 and 2, were used for each stream to provide both riffle-like and pool-like environments. Six such streams were used. The water in each upper

Figure 1. General view of the recirculating artificial stream apparatus.

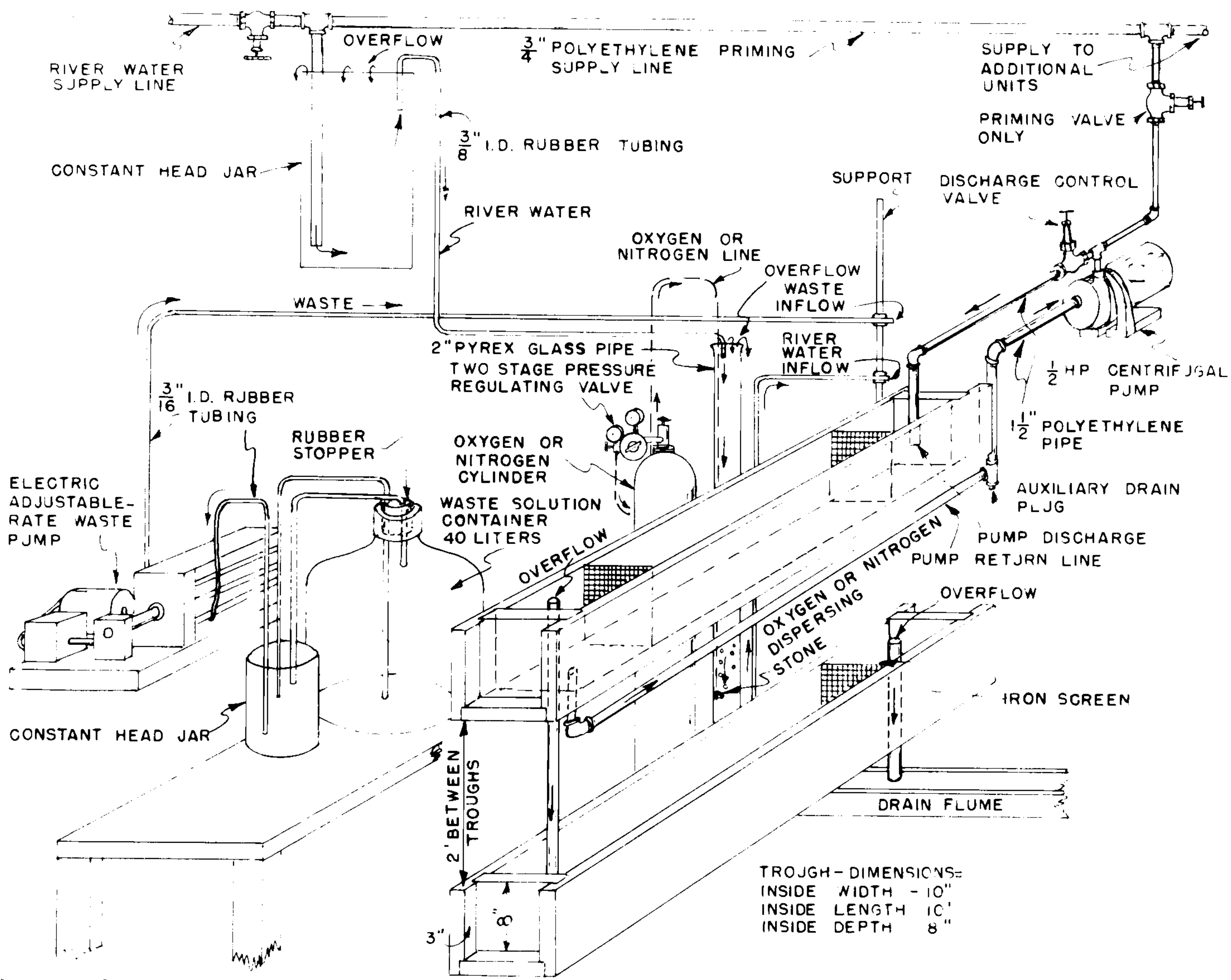

Figure 2. Diagrammatic representation of one unit of the artificial stream apparatus.

trough could be recirculated separately by means of a 1/2 h.p. pump so as to produce a current. The water from the upper trough of each stream overflowed into the lower trough, which had no appreciable current, thus resembling a pool. Some of the pool experiments to be discussed were carried on in these lower troughs. Each pair of upper troughs was lighted by a fluorescent unit.

The velocity of the current in the upper troughs could be controlled by means of valves at the outputs of the pumps and by varying the water depth. A surface velocity of about one-half foot per second was maintained in all "riffle" experiments. Surface velocities were determined by the "float" method and a current meter was used for a few measurements of sub-surface velocities. The pump output entering each upper trough at one end was directed downward. An arrangement of baffles, not shown in Figure 2, directed a rather even current horizontally over the stream bottom materials. The rate of introduction of new river water into each upper trough was controlled at about 1,500 ml. per minute in most experiments by adjusting the height of the discharge point of tubing coming from an over flowing, constant-head jar. In some experiments only unpolluted river water flowed through the troughs. In others various suitable dilutions of pulp mill wastes were introduced at the rate of about 10 ml. per minute from 12-gallon bottles by means of chemical pump, so as to maintain desired waste concentrations.

In some experiments, it was desired to produce and maintain a concentration of dissolved oxygen different from that which would otherwise prevail. To accomplish this, the fresh water flowing into a trough was first passed down a glass column packed with Raschig rings, as shown in Figure 2. When it was desired to increase the oxygen concentration, a controlled flow of oxygen bubbles was passed upward through the column. A two-stage pressure-reducing valve was used for regulating the flow of the gas. When it was desired to maintain a low concentration of oxygen in the absence of waste, nitrogen was bubbled through the water in the column, thus removing oxygen from the water.

Arrangement of bottom materials

Bottom materials for the troughs which served as the artificial stream beds were selected partly on the basis of reproducibility and workability. Reproducibility was important in standardizing conditions among troughs and experiments. Workability involved the selection of materials which enabled accurate quantitative measurements of trough fauna with a reasonable expenditure of effort.

Stream bottom aggregate was screened and several size-classes of gravel were established. Various mixtures of the different classes were manipulated in the troughs and compared with respect to their workability.

Another consideration in the selection of bottom materials and their arrangement was their suitability as components of the environment of the flora and fauna to be studied. There must be a large number of factors which are important in this respect. For example, particle size was important in the selection by caddisfly larvae of materials for use in larval case construction. Caddisfly larvae of the family Limnephilidae in troughs containing bottom materials that included gravel less than 1/4 inch in diameter closed the open end of their larval tubes with pieces of the finer gravel before pupation. In troughs containing only large or intermediate sizes of particles, the larval cases were usually not closed, except possibly by larval secretions and detritus. Particle size also has much to do with the pattern and speed of water flow through the bottom materials and utilization of the spaces between these materials by animals. Caddisfly larvae pupated well down in the interstices in the fine gravel aggregate. Gravel of this size must be of considerable protective value in this way to the pupae in a natural system. The caseless larvae of Hydropsyche, a caddisfly, most frequently constructed their nets in the interstices between the layer of rubble and the underlying substrate of fine gravel. Some emerging insects utilized the rubble extending above the water surface, but many others successfully emerged on the sides of the troughs and on the screens at the lower ends of the troughs.

Three "standard" types of bottom, as follows, were established, and used in various experiments and preliminary screening:

Bottom type #1 - rubble (rocks about 4 to 8 inches in diameter) only.

Bottom type #2 - rubble plus fine gravel (which passed through a one-inch mesh screen, but was held by a one-half-inch mesh screen) in a ratio of 7 parts rubble to 3 parts gravel, by volume. See Figure 3.

Bottom type #3 - rubble plus fine gravel plus smaller gravel (which passed through a one-fourth-inch mesh screen, but was held by a 3/32 inch screen) in a ratio of 2:1:1 parts by volume, respectively.

<u>Selection and use of test animals</u>

There are numerous considerations in the selection of animals for long-term studies. Where considerable numbers of animals are required, they must be readily available in sufficient quantity during all seasons in

Figure 3. The rubble and fine gravel used as stream bottom materials; bottom type #2.

which the tests are to be conducted. The animals must be large enough for easy enumeration and for easy identification in the field and in the trough during an experiment.

A variety of insects, crustaceans, and molluscs were collected and held under laboratory conditions so that their suitability as test animals could be studied. Ready availability in sufficient numbers of suitably large forms during one or more seasons of the year narrowed down the main possibilities to some stonefly naiads, caddisfly larvae, crayfish, and snails, although mayfly naiads were used in some experiments. The forms used included:

Class Crustacea

Subclass Malacostraca

Order Decapoda

Pacifastacus trowbridgi (Stimpson), crayfish

Class Insecta

Order Ephemeroptera (mayflies)

Isonychia sp.

Order Plecoptera (stoneflies)

Pteronarcys sp.

Acroneuria californica (Banks)

Acroneuria pacifica Banks

Order Trichoptera (caddisflies)

Hydropsyche sp.

Unidentified limnephilid

Class Gastropoda

Subclass Pulmonata

Physa sp., pond snail

Subclass Prosobranchia

Fluminicola sp., stream snail

Goniobasis sp., stream snail

Class Teleostomi

Order Clupeiformes

Oncorhynchus kisutch (Walbaum), coho salmon

Oncorhynchus tshawytscha (Walbaum), chinook salmon

Salmo gairdneri gairdneri (Richardson), steelhead trout

Order Cyprinodontiformes

Lebistes reticulatus (Peters), common guppy

Order Perciformes

Micropterus salmoides (Lacepede), largemouth bass

Cottus perplexus (Gilbert and Evermann), reticulate sculpin

Generally, collections were made with a small minnow seine three or four days before the beginning of an experiment. The animals were held in aerated stream or pond water in glass jars at a temperature near that expected in the forthcoming experiment. Generally, there was about a 10 per cent loss of stonefly naiads of the genus Acroneuria in the first 24 hours of holding. After this initial loss, mortality leveled off at perhaps one or two per cent per day, and most of this was attributed to cannibalism. Some stonefly naiads, snails, crayfish, and caddisfly larvae were successfully held in the jars without excessive mortality for several months at a time. The incidence of cannibalism and predation appeared to be related to the abundance of smaller food organisms and the amount of vegetation contained in the jars. Schoenemund (40) also observed cannibalism among stoneflies in containers, but attributed most losses, especially among the more oxygen-sensitive species, to insufficiency of dissolved oxygen, except when special precautions were made to provide oxygen concentrations near saturation. In my work with Acroneuria pacifica and A. californica, the oxygen concentration in the stock animal containers appeared not to be so critical, as on several occasions naiads of these species lived in jars for several weeks without apparent distress in water that was not artificially oxygenated.

In this thesis, the name "Acroneuria" refers to both Acroneuria californica and Acroneuria pacifica unless the particular species is indicated. In many stream and pool experiments it was necessary to combine the two species in order to have enough animals with which to conduct an experiment. All test animals for the artificial stream work were collected within about 50 miles of Corvallis, Oregon.

Ordinarily two or three days before commencing the flow of waste liquor in an experiment, the test animals were counted into the artificial streams and allowed to adjust themselves to some degree to the new environment. Stonefly naiads, snails, and encased caddisfly larvae ceased rapid exploratory movements in a few hours. Several days were required for net-building caddis larvae to occupy suitable locations and construct nets. Observations of the condition and activities of the test animals were made frequently during the experiments. As dead specimens appeared they were removed from the troughs and the dates were recorded. Generally, a complete inventory of all animals in a trough was not made until the termination of an experiment. Unnecessary movement of bottom materials may destroy the nets of caseless caddisfly larvae, crush fragile larvae, loosen bacterial and algal growths, and severely alter the environment at certain stages of community development.

An inventory of test animals could be made rapidly under control conditions, but in the presence of dense masses of the sheathed bacterium Sphaerotilus natans, which were produced under some test conditions, enumeration was greatly slowed. Under such conditions of heavy "slime" growth, an accurate count of very small test animals is not feasible.

The final inventory was made by examination and washing of each individual large rock and almost every individual piece of fine gravel in each trough. The material in each trough was gone over three or four times. Experiments with a known number of individuals indicated that two or three complete examinations of all bottom materials were necessary to achieve a complete enumeration of larger organisms. The numbers and general condition of the animals found were recorded. Only those animals which showed no movement whatsoever on probing over a period of several minutes were considered dead.

Periphyton methods

Descriptive records were kept of bacterial slime growth on the bottom materials and the walls of the troughs. The criteria for these records were as follows:

(1) Light growth - spotty or indistinct *Sphaerotilus natans* growth; strands generally less than one-half inch in length (see Figure 4).

(2) Medium growth - distinctly evident *Sphaerotilus* growths on all or most visible surfaces; many *Sphaerotilus* strands one inch in length (see

Figure 4).

(3) Heavy growth - lush *Sphaerotilus* growths covering all initially visible underwater rock and trough surfaces, but the outline of individual rocks still evident. Usually many strands over two inches long (see Figure 4).

(4) Secondary bottom - lush *Sphaerotilus* growth (plus associated materials) on all initially visible underwater surfaces, forming a continuous blanket over the bottom and obscuring the outlines of individual rocks and the sides of the troughs (see Figures 4 & 5).

Sphaerotilus growths were identified with the assistance of descriptions and photographs in Stokes (43, p. 279-291) and Pringsheim (37, p. 453-482).

Sulfite waste liquor and its standardization

Sulfite waste liquor is produced by a pulp manufacturing process involving the action of bisulfites of calcium, magnesium, or ammonia on wood chips in the presence of excess sulfurous acid, aided by heat and pressure (Gaudy 21, p. 37). After recovery of the pulp on screens, a strongly acid fluid remains that contains numerous and variable components. Although some of the sulfite liquor is reclaimed, the effluents from sulfite mills include the

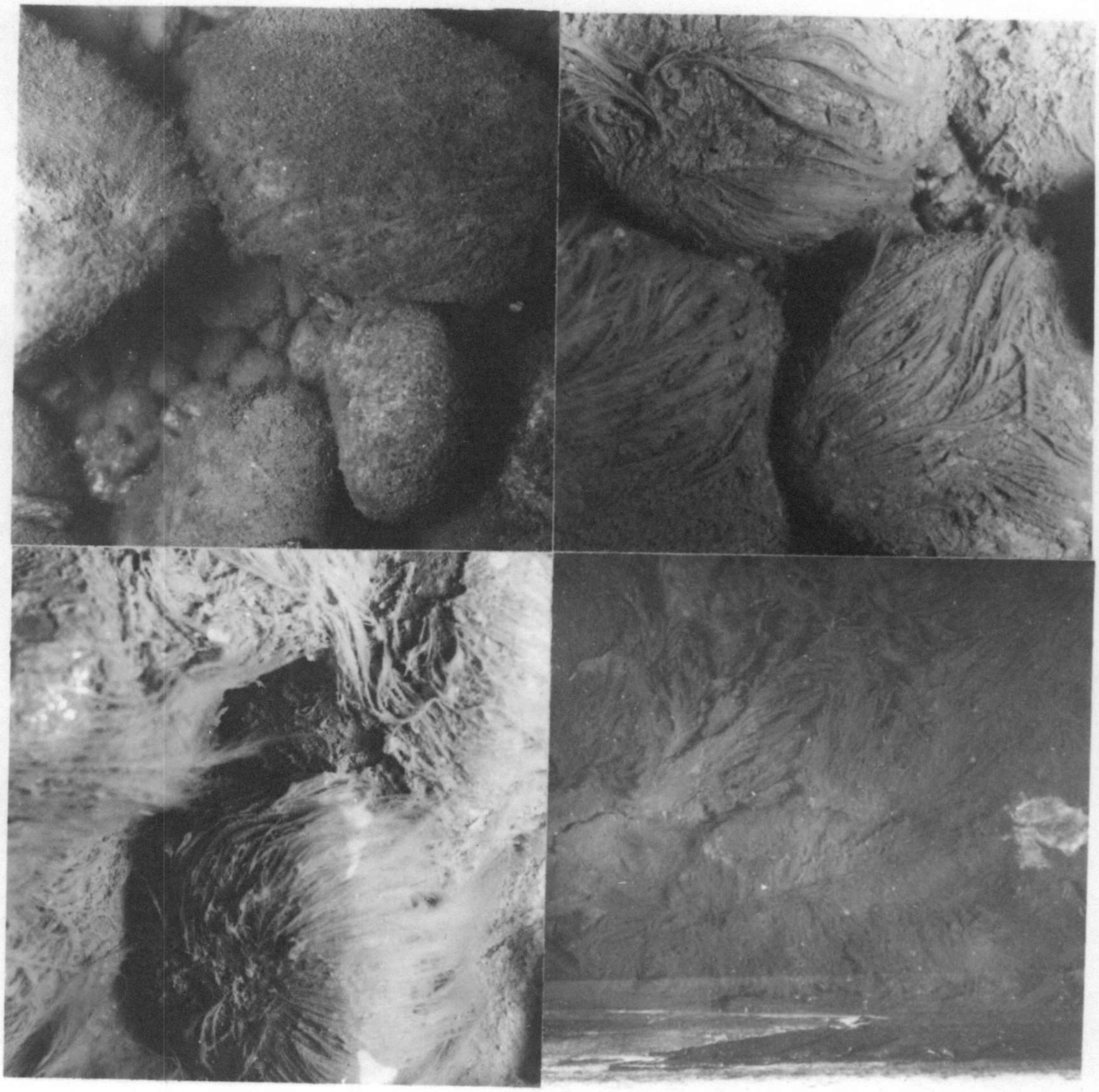

Figure 4. Catagories of growth of Sphaerotilus natans. Upper left - light growth; upper right - medium growth; lower left - heavy growth; lower right - secondary bottom.

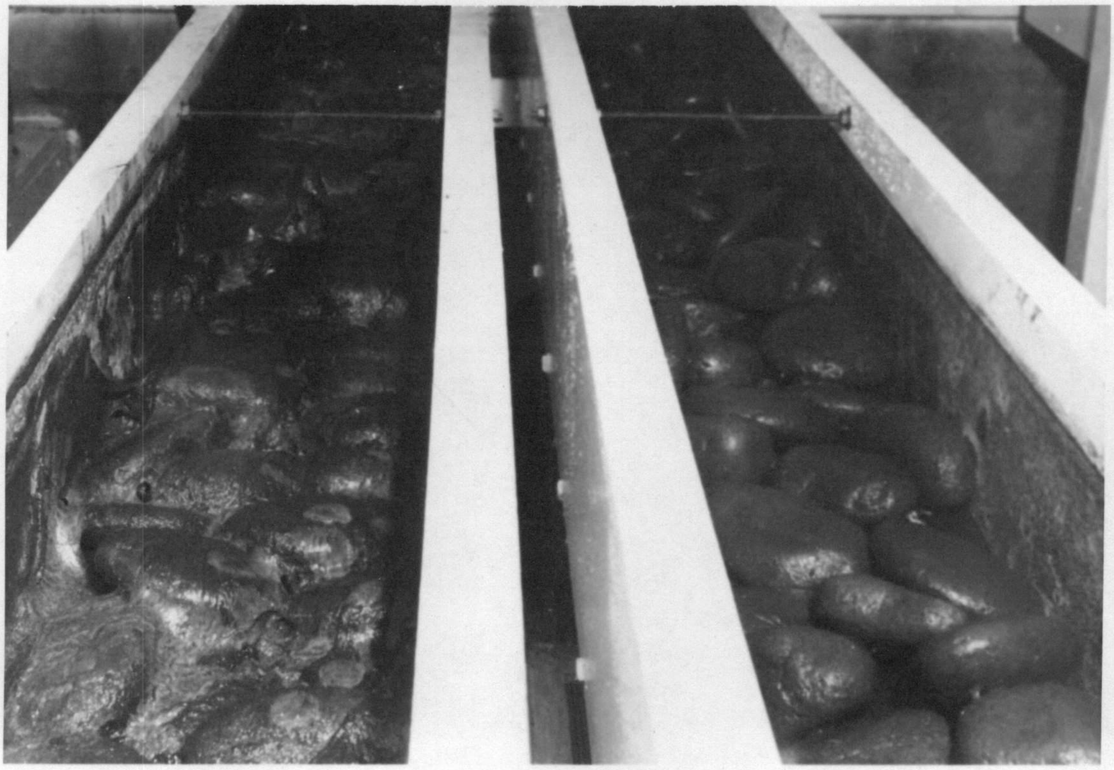

Figure 5. A comparison of a secondary bottom produced by S. natans and a "clean" bottom after removal of the water.

liquor, various wash waters used in removing the liquor from the pulp, a large amount of wood extractives, and some pulp. The waste used in this study no doubt contained much less fiber than is ordinarily present in mill effluents. Thus, wood fiber was not an environmental factor in these experiments as it may be in natural waters receiving mill wastes (Gunter and McKee, 23, p. 27, Williams, et al., 54, p. 8).

The sulfite waste used for the artificial stream experiments was calcium-base sulfite waste liquor of digester strength, and this was transported and stored in closed 5-gallon glass bottles. The total dried solids content of each sample of waste was determined, and the dilutions to be introduced into the streams were prepared once a day, their solids content being adjusted so as to provide for the desired rate of introduction of waste into each stream. The waste concentrations maintained in the artificial streams are expressed in this paper as mg/l sulfite waste liquor with a total dried solids content of 10 per cent. Thus, when the concentration maintained in a stream was equivalent to 30 mg/l dried sulfite waste solids, the concentration is expressed in this thesis as being equivalent to 300 mg/l sulfite waste liquor having 10 per cent total solids.

Whether the concentrations of biologically active components of calcium-base sulfite waste liquor are or are not very nearly proportional to the total solids content of the waste cannot be stated with confidence. The absence of any acute toxicity to aquatic animals of sulfite waste liquor at concentrations approaching those tested in the artificial streams and those usually occurring in waters receiving this waste would render meaningless any standardization of the waste on the basis of toxicity as measured by short-term bioassay. Biochemical oxygen demand determinations might provide a useful means for standardizing these wastes according to their relative ability to promote bacterial growth and cause reduction of dissolved oxygen. Probably the total solids content of the sulfite waste liquor samples used for these experiments was in a general way proportional to the concentrations of the components of main biological importance in the artificial streams. Standardization of the waste samples on the basis of their solids content no doubt resulted in greater uniformity of stream conditions in corresponding tests than that which could be achieved by diluting the wastes on a strictly volumetric basis without regard to the solids content of the different waste samples used.

Kraft waste liquor and its standardization

Kraft paper pulps are products of a process wherein a "white-liquor" mixture primarily of sodium sulfate, sodium sulfide, sodium carbonate, and sodium hydroxide is used as a digestant for wood chips (Carlin, 8, p. 124, 125). After removal of the pulp, a waste fluid called "black liquor" remains which is alkaline and carries a variety of compounds resulting from disintegration of the wood. As kraft pulps are usually not cooked out as completely as sulfite pulps, the organic content of the black liquor is generally not as high as that of sulfite liquor. Black liquor is not ordinarily an important constituent of kraft mill effluents. The cooking liquor and also concentrated pulp washings containing residual black liquor are normally evaporated for recovery of chemicals, the resulting steam is condensed, and the organic residue burned. Condensates resulting from this and other operations are discharged as components of the effluent, along with dilute pulp washings and various other process wastes of minor biological importance (usually including large volumes of paper machine white water).

The "whole" prepared kraft waste used was composed of combined condensates resulting from the process which recovers reusable chemicals from the black liquor and from other sources, plus some of the black liquor. The mixture finally used was 99 per cent combined condensates and 1 per cent black cooking liquor. The black liquor solids in a mill effluent are contributed largely by waste water which has been used for washing pulp, but black liquor was used in place of the dilute wash water in preparing the kraft mill waste for use in the experiments. The prepared "whole" kraft waste was not as dilute as would be the whole effluent of a typical kraft process mill which would usually contain large amounts of pulp wash water, paper machine water, and water from other sources. The waste source was a mill pulping Douglas fir.

Rather large quantities of waste were necessary for the artificial stream experiments, and the problem of transporting waste to the laboratory was lessened by using a concentrated rather than a dilute waste. Also, the prepared concentrated waste doubtless was more uniform in composition than the more dilute mill effluent. The prepared waste was believed to contain combined condensates and black liquor solids in approximately the same proportions to each other in which they would occur in a typical though more dilute mill effluent. Recent unpublished qualitative and quantitative information on the toxic constituents of kraft mill wastes indicates, however, that no dilution of black liquor is quite comparable in composition to pulp wash water, and that the amount of black liquor used in the prepared waste was not sufficient for introducing the proper amounts of certain toxicants to be found in the wash water.

Since several batches of waste are necessary to complete a single artificial stream experiment and the batches vary in toxicity, adjustment of the waste concentration maintained in the streams is necessary from batch to batch if the toxicity is to remain constant throughout the experiment. The 24-hour median tolerance limit (24-hour TL_m) at 20°C. for young guppies (Lebistes reticulatus) or the sculpin (Cottus perplexus) of each batch of prepared kraft waste was determined before that batch was used. A concentration of waste that was some fixed fraction of the 24-hour TL_m was then maintained in each stream during the entire experiment, regardless of the rate of introduction of a batch necessary to maintain the toxicity-adjusted concentration. Thus, for example, when the 24-hour TL_m of a waste sample was 4.0 per cent by volume, concentrations equal to 0.025, 0.05, 0.1, 0.2, and 0.3 times this value (or 0.1, 0.2, 0.4, 0.8, and 1.2 per cent waste by volume) were maintained in five of the artificial streams (the sixth receiving no waste and serving as a control) until a new batch of waste had to be used. If the next batch of waste was found to be twice as toxic, the

24-hour TL_m being 2.0 per cent, the waste concentrations in all streams were adjusted accordingly, ranging from 0.025 x 2.0 per cent to 0.3 x 2.0 per cent (or 0.05 to 0.6 per cent by volume). The waste concentrations maintained in the streams were thus adjusted to the acute toxicity characteristics of each batch. It must be recognized, however, that the manner of harmful action of a complex toxic waste on an organism is not always the same at rapidly fatal as at slowly fatal concentrations, and there is the possibility that the factors producing long-term effects of the waste may not have been kept constant by the adjustments based on acute toxicity. There is the further possibility that the mode of action of a waste on different kinds of organisms might not be the same, and concentration adjustments based on the acute toxicity to one kind of organism might not necessarily insure constant conditions for all kinds of organisms from batch to batch. This standardization procedure has been described by Warren and Doudoroff (51, p. 211A-216A) and they discuss in some detail its limitations. The general procedure for bioassays was based on the recommendations of Doudoroff, et al. (12, p. 1380-1397).

A single batch of waste, when used for an artificial stream experiment, would usually be enough to last from one to two weeks. The combined condensate was transported and stored in closed glass jars. Condensates, on storage in closed glass containers, generally tend to lose toxicity, some batches losing toxicity faster than others. Both the combined condensates and the black cooking liquor were stored at full strength. It is unlikely that the cooking liquor, being 10 to 12 per cent solids, changed much in its characteristics on storage for a short time. The prepared waste solutions which were introduced into the streams contained combined condensates, black liquor, and enough water to adjust the solutions to the desired strength for the necessary rate of introduction. These diluted wastes probably changed much more rapidly than did the stored concentrated wastes, and the solutions being introduced were consequently prepared almost daily. A sample of prepared waste made from fresh batches of combined condensate and black liquor had a 24-hour TL_m for Cottus perplexus of 2.9 per cent, while a sample prepared from these same batches when 14 days old had a TL_m of 3.1 per cent. Prepared waste made from other batches of combined condensate and black liquor which were five days old had a 24-hour TL_m for the guppy of 4.4 per cent. Waste prepared from this condensate and black liquor when the latter were 12 days old had a 24-hour TL_m of 4.6 per cent and when they were 19, 26, and 54 days old the 24-hour TL_m's for the prepared wastes were, respectively, 5.1 per cent, 6.2

per cent, and 6.5 per cent. Usually, batches of waste were completely used before they were two weeks old and it is not believed that changes in toxicity on storage for this length of time were an important source of error in the artificial stream experiments.

Toxicity standardization of the different batches of prepared kraft wastes for the first artificial stream experiment was accomplished by using Cottus perplexus for determining the 24-hour TL_m's. Guppies approximately one week old were used for later standardizations. The more uniform age, size, environmental history and genetic background of the guppies and their smaller size made them a more desirable test animal for standardization. Guppy bioassays were performed in 1-gallon, widemouth jars containing 3 liters of test solution. Bioassays with Cottus perplexus and other species of fish were performed in 5-gallon wide-mouth jars containing 15 liters of test solution. In the bioassays with guppies and Cottus perplexus, solutions were renewed at the end of 12 hours to maintain the dissolved oxygen above 5 mg/l, and also to prevent an excessive decline of the concentration of any toxicants in the test solutions. All bioassays were performed in a constant temperature room held at 20°C.

The 24-hour TL_m's for guppies of 15 different prepared kraft samples, which were used for the second through the sixth artificial stream experiments with kraft waste, are given in Table 1. An attempt was made to use 20 fish at a concentration for estimating these TL_m's. However, sufficient fish were not always available for this, and some therefore less precise estimates were based on smaller numbers. The total oxidizable sulfur, total chemical oxygen demand, and biochemical oxygen demand of some of the prepared waste samples are given in Table 1, along with the TL_m values reported. The 24-hour TL_m for Cottus perplexus of five waste samples prepared for the first kraft waste experiment are given in Table 2. The 24-hour, 48-hour, 72-hour, and 96-hour TL_m's for Cottus perplexus of a sample of prepared kraft waste were determined, and these were 2.8 per cent, 2.1 per cent, 1.7 per cent, and 1.4 per cent, respectively.

When a single species is used for performing standardization bioassays, comparable data on the tolerance of that species and of other species of interest are instructive. Some reference data are presented in Table 3 on the acute toxicity of prepared kraft waste samples to various fishes and the pond snail. Bioassays with coho salmon, steelhead trout, and largemouth bass were performed in 15 liters of test solution, and the dissolved oxygen was maintained near saturation by passing 50 to 70 bubbles per minute of oxygen gas through the test

Table 1. Toxicity at 20° and Chemical Characteristics of Samples of Prepared Kraft Waste.

Sample Number	Sample Date	Guppy 24-hr. TL_m Per Cent	Number of Fish Per Container	Total Oxidizable Sulphur mg/l	Total Chemical Oxygen Demand mg/l	Biochemical Oxygen Demand mg/l
1	6/14/56	4.35	20	372	2770	1118
2	7/16/56	8.73	20	443	2629	1033
3	7/30/56	8.65	20	418	2660	--
4	8/13/56	3.20	20	342	3320	1330
5	8/27/56	6.10	20	453	2596	1000
6	9/15/56	2.17	20	576	--	--
7	10/11/56	1.70	20	418	2898	1170
8	10/18/56	2.80	10	512	3218	1390
9	10/26/56	3.20	10	--	--	--
10	2/9/57	2.60	10	--	--	--
11	3/6/57	4.60	10	--	--	--
12	3/15/57	3.55	10	--	--	--
13	6/13/57	8.70	10	--	--	--
14	6/24/57	4.85	10	--	--	--
15	6/26/57	7.00	5	--	--	--

Table 2. Toxicity of Samples of Prepared Kraft Waste to *Cottus perplexus* at 20°C.

Sample Letter	Sample Date	No. Fish per Concentration	24-Hour TL_m
A	7/17/55	10	4.60
B	7/21/55	10	3.83
C	7/29/55	10	2.93
D	8/4/55	10	3.20
E	8/22/55	10	3.48

Table 3. Relative Toxicity of Prepared Kraft Waste to Various Test Animals at 20°C.[1]

Species	24-Hour Median Tolerance Limit in Per Cent					
Waste Sample No.	1	3	4	5	6	7
Guppy[2]	4.35	8.65	3.20	6.10	2.17	1.7
Sculpin[2]	-	-	-	-	-	1.7[4]
Coho Salmon[3]	3.30	-	4.75	7.50	-	-
Steelhead Trout[3]	-	-	-	5.95	3.70	-
Largemouth Bass[3]	-	-	-	11.10	-	-
Pond Snail[2]	-	14.75	8.45	12.75	-	-

(1) 20 animals per concentration.

(2) Solutions renewed at 12 hours.

(3) Solutions not renewed but oxygen used.

(4) 10 animals per concentration.

solutions, the solutions not being renewed at the end of 12 hours. The 24-hour TL_m of coho salmon for a sample of prepared waste was determined to be 4.2 per cent by the solution-renewal method and 5.6 per cent by the oxygen-bubbling method. The solution-renewal method was used for the pond snail bioassays.

RESULTS OF ARTIFICIAL STREAM EXPERIMENTS

Experiments with sulfite waste liquor

Artificial stream experiments were performed to determine some of the effects on various aquatic organisms of concentrations ranging from 300 to 1,200 mg/l calcium base sulfite waste liquor having 10 per cent solids. Concentrations as high or higher than these are sometimes found (Williams, et al., 54, p. 18) but would not usually be expected in receiving waters except in the vicinity of waste outfalls. These high concentrations were maintained in order to facilitate the detection of any toxicity of the waste and to make possible the study of the influence on the benthic environment and on benthic organisms of the development of large quantities of Sphaerotilus natans. Low dissolved oxygen concentration, resulting chiefly from bacterial decomposition of waste components, was another one of the factors the biological significance of which required study. Artificial stream studies of the effects of sulfite waste concentrations much lower than 300 mg/l will be necessary in the future if their influence on the productivity of important aquatic organisms, and not only on their survival is to be adequately evaluated. Table 4 summarizes the general conditions and some results of all pool and stream experiments with sulfite waste.

Percentages of survival of the various test animals were determined after exposure for periods ranging from 14 to 35 days to various concentrations of sulfite waste in artificial riffle and pool environments. Table 5 summarizes the survival of these animals in all experiments with sulfite waste. The most important results were obtained with the stoneflies *Acroneuria pacifica* and *Acroneuria californica*. Although the variability in survival percentages of *Acroneuria* was high at each of the waste concentrations studied, as shown in Figure 6, there can be little doubt that survival even at the concentration of only 300 mg/l averaged significantly less than in control tests. With increasing concentration, survival tended to decrease, though even at the concentration of 1,000 mg/l relatively high survival percentages sometimes were observed. Control survival percentages were not high, and they ranged from 50 to 85 per cent. The death of controls is believed to have been due largely to cannibalism or predation of one species of *Acroneuria* on the other. Figure 6, which shows survival percentages generally declining with increasing waste concentration, can be interpreted only as illustrating the total effect of all survival depressing processes occurring in the presence of the waste. Whether the lower survival

Table 4. Summary of Conditions and Some Results of Experiments with Sulfite Waste Liquor.

Exper. Number	Incl. Dates	Length in Days	Type	Bottom Type	Approx. Water Inflow ml/min.	Surf. Veloc. ft/sec.	Concen. Liquor in mg/l	Temp. Range °C.	Range of Recorded Dissolved Oxygen[1] Above Rocks Min.	Above Rocks Max.	Under Rocks Min.	Under Rocks Max.	Slime Develop.	Slime and Debris[2] c.c.	Oxygen-ated
I	Sept. 10-	(30)	pool	1	1400	nil	1000	12.8-21.1	4.2	-	3.3	5.4	heavy	2500	no
	Oct. 10,		do	do	do	do	600		4.6	-	4.0	6.4	medium	2500	do
	1955		do	do	do	do	300		5.2	-	4.3	6.4	light	2200	do
			do	do	do	do	control		6.8	8.2	5.8	-	none	1200	do
II	Nov. 5-	(14)	pool	1	1400	nil	1000	3.9-12.8	5.8	7.1	-	-	heavy	1550	do
	Nov. 19,		do	do	do	do	600		6.0	10.1	-	-	medium	1390	do
	1955		do	do	do	do	300		7.0	-	-	-	medium	1350	do
			do	do	do	do	control		7.8	-	-	-	none	600	do
III	Apr. 4-	(31)	pool	1	1400	nil	1000	6.7-15.6	2.0	6.5	0.0	1.3	sec. bot.	4520	do
	May 5,		do	do	do	do	600		-	7.6	2.1	4.3	heavy	3500	do
	1956		do	do	do	do	300		-	8.1	3.4	6.7	medium	2050	do
			do	do	do	do	control		8.9	9.3	-	-	none	730	do
IV	May 19-	(14)	pool	1	1400	nil	1000	13.4-18.9	8.2	10.0	5.6	10.0	medium	-	yes
	June 2,		do	do	do	do	1000		5.8	6.2	5.2	6.0	heavy	-	no
	1956		do	do	do	do	300		6.8	7.6	5.6	7.6	light	-	do
			do	do	do	do	control		7.6	8.6	7.6	8.6	none	-	do
V	June 22-	(14)	riffle	2	1500	0.4	800	22.3-24.5	2.6	5.0	0.6	4.7	medium	3000	do
	July 5,		do	do	do	do	800		4.2	7.7	4.0	6.9	medium	3000	yes
	1957		do	do	do	do	control		5.7	6.1	-	-	none	450	no
			do	do	do	do	control		7.8	8.2	-	-	none	400	do
			pool	no rocks	do	nil	800		3.8	5.0	-	-	-	-	do
			do	do	do	do	800		6.3	6.5	-	-	-	-	yes
			do	do	do	do	control		5.7	6.6	-	-	-	-	no
			do	do	do	do	control		7.8	7.9	-	-	-	-	do
VI	July 16-	(14)	riffle	2	1500	0.4	1200	21.7-25.0	5.4	6.4	2.4	7.3	medium	4200	yes
	July 29,		do	do	do	do	1000		4.0	7.6	3.4	7.6	medium	2500	yes
	1957		do	do	do	do	800		1.9	5.0	1.4	4.8	medium	2100	no
			do	do	do	do	600		3.0	4.4	2.4	4.4	medium	2000	do
			do	do	do	do	control		5.3	5.6	-	-	none	800	do
			do	do	do	do	control		7.0	7.5	-	-	none	750	do
			pool	no rocks	do	nil	600		2.5	4.2	-	-	-	-	do
			do	do	do	do	800		2.3	4.2	-	-	-	-	do
			do	do	do	do	1000		4.4	4.7	-	-	-	-	yes
			do	do	do	do	1200		3.7	4.3	-	-	-	-	yes
			do	do	do	do	control		5.4	6.0	-	-	-	-	no

1 Lowest and highest dissolved oxygen concentrations, in mg/l, determined with the Alsterberg (azide) modification of the Winkler method. Where no maximum value is given to complement minimum recorded values, concentrations near the probable maximum were not determined.

2 Thirty-minute settled volume in 5-gallon jars of washings of trough and all bottom materials at end of experiment.

43

Table 5. Survival of Test Animals in All Experiments with Sulfite Waste Liquor.

Exper. No.	Length in Days	Test Animal	1200 mg/1			1000 mg/1			800 mg/1			600 mg/1			300 mg/1			Control A			Control B			Oxygenated
			In	Out	Per Cent Survival	In	Out	Per Cent Survival	In	Out	Per Cent Survival	In	Out	Per Cent Survival	In	Out	Per Cent Survival	In	Out	Per Cent Survival	In	Out	Per Cent Survival	
I	(30)	A. pacifica and A. californica	-	-	-	40	7	17.5	-	-	-	40	18	45.0	40	19	47.5	40	34	85.0	-	-	-	no
		Pteronarcys sp.	-	-	-	3	1	33.3	-	-	-	3	3	100.0	3	1	33.3	3	2	66.7	-	-	-	do
		Fluminicola sp.	-	-	-	30	29	96.7	-	-	-	30	27	90.0	30	30	100.0	30	30	100.0	-	-	-	do
		Goniobasis sp.	-	-	-	30	29	96.7	-	-	-	30	30	100.0	30	30	100.0	30	28	93.3	-	-	-	do
		P. trowbridgi	-	-	-	10	8	80.0	-	-	-	10	9	90.0	10	7	70.0	10	10	100.0	-	-	-	do
II	(14)[1]	A. pacifica and A. californica	-	-	-	40	18	45.0	-	-	-	40	21	52.5	40	(18)[2]	45.0	40	24	60.0	-	-	-	do
		Pteronarcys sp.	-	-	-	3	2	66.7	-	-	-	3	3	100.0	3	2	66.7	3	3	100.0	-	-	-	do
		P. trowbridgi	-	-	-	12	11	91.7	-	-	-	12	11	91.7	12	10	83.3	12	11	91.7	-	-	-	do
		Goniobasis sp.	-	-	-	30	29	96.7	-	-	-	30	30	100.0	30	30	100.0	30	30	100.0	-	-	-	do
III	(31)	A. pacifica and A. californica	-	-	-	40	0	0.0	-	-	-	20	(4)[2]	20.0	20	(4)[2]	20.0	20	(13)[2]	65.0	-	-	-	do
IV	(14)	A. pacifica and A. californica	-	-	-	20	(13)[3]	65.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	yes
		do	-	-	-	20	(5)[3]	25.0	-	-	-	-	-	-	20	(7)[3]	35.0	20	(17)[3]	85.0	-	-	-	no
		Limnephilid larvae[4]	-	-	-	20	12	60.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	yes
		do	-	-	-	20	2	10.0	-	-	-	-	-	-	20	3	15.0	20	2	10.0	-	-	-	no
V	(14)	A. pacifica and A. californica	-	-	-	-	-	-	30	4	13.3	-	-	-	-	-	-	30	15	50.0	30	20	66.7	no
		do	-	-	-	-	-	-	30	19	63.3	-	-	-	-	-	-	-	-	-	-	-	-	yes
		Pteronarcys sp.	-	-	-	-	-	-	5	0	0.0	-	-	-	-	-	-	5	3	60.0	4	3	75.0	no
		do	-	-	-	-	-	-	5	3	60.0	-	-	-	-	-	-	-	-	-	-	-	-	yes
		Limnephilid larvae[5]	-	-	-	-	-	-	60	2	3.3	-	-	-	-	-	-	60	51	85.0	60	48	80.0	no
		do	-	-	-	-	-	-	60	45	75.0	-	-	-	-	-	-	-	-	-	-	-	-	yes
		P. trowbridgi	-	-	-	-	-	-	10	0	0.0	-	-	-	-	-	-	-	-	-	-	-	-	no
		do	-	-	-	-	-	-	10	2	20.0	-	-	-	-	-	-	-	-	-	-	-	-	yes
VI	(14)	A. pacifica and A. californica	60	3	5.0	30	5	16.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	yes
		do	-	-	-	-	-	-	30	1	3.3	30	4	13.3	-	-	-	20	14	70.0	20	14	70.0	no
		Pteronarcys sp.	-	-	-	8	4	50.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	yes
		do	-	-	-	-	-	-	8	0	0.0	-	-	-	-	-	-	-	-	-	-	-	-	no
		Limnephilid larvae[5]	60	2	3.3	60	2	3.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	yes
		do	-	-	-	-	-	-	60	0	0.0	60	0	0.0	-	-	-	60	58	96.7	60	54	90.0	no
		P. trowbridgi	-	-	-	16	3	18.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	yes
		do	-	-	-	-	-	-	16	0	0.0	-	-	-	-	-	-	-	-	-	-	-	-	no

1 Test ended by breakdown and disruption of conditions on 15th day.
2 High mortality probably was due to failure of water supply.
3 This number includes those known to have successfully emerged, but probably not all emergents were observed, so that actual survival may have been greater.
4. Larvae from Santiam River
5 Larvae from Alsea River

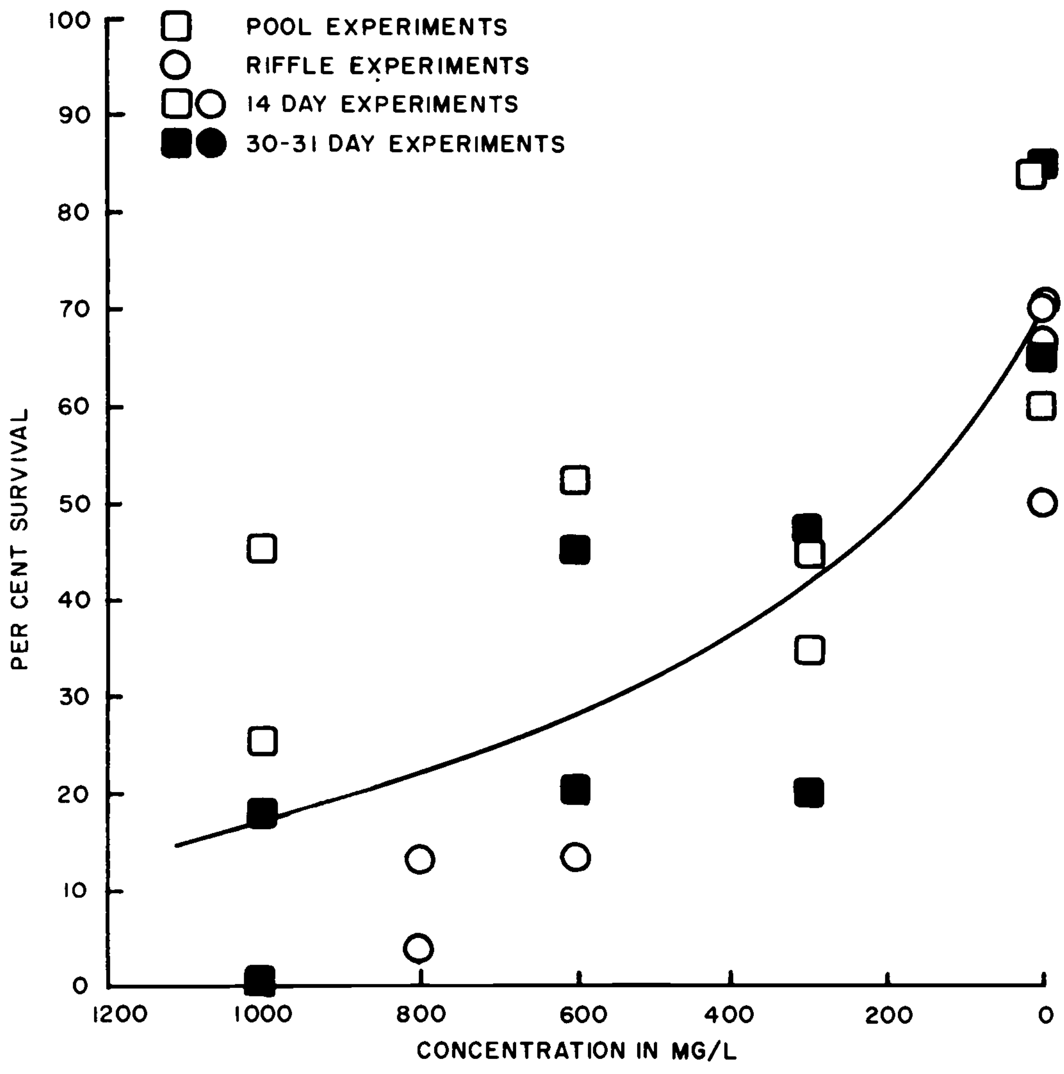

Figure 6. The survival of Acroneuria at different concentrations of sulfite waste liquor.

percentages are due primarily to toxic action, to the presence of large amounts of benthic growth, to the lowering of the dissolved oxygen concentration, or to the interaction of these or other processes, is not indicated by any relationship such as that represented by Figure 6.

Before an attempt is made to distinguish between possible toxic action of the waste, the effects of lowered oxygen levels, and other possible survival-depressing actions or interactions, it is advisable to consider, among other things, the sequence of development of Sphaerotilus natans growths and their impact on benthic environmental conditions, particularly with regard to dissolved oxygen. Within a period of about 10 days, at the lower sulfite waste concentrations, a light growth of Sphaerotilus usually developed in the artificial streams (Figure 4); whereas at the higher concentrations a medium or heavy growth developed (Figure 4). Under some conditions, the growth was so heavy as to form a secondary bottom (Figure 4), thus obstructing exchange of water between the superficial layer of water and the water occupying the spaces among the rocks inhabited by benthic organisms. The sequence of Sphaerotilus development resulting from the presence of sulfite waste is much the same as that of growth resulting from the introduction of kraft waste. Data from a kraft waste experiment, rather than a sulfite waste experiment, are introduced here because they provide the most graphic example of certain relationships of interest. The general levels of *Sphaerotilus* development on successive days at a rather high concentration of kraft waste are given in Figure 7. Also shown in Figure 7 are the dissolved oxygen concentrations in the control stream and in the stream receiving the kraft waste. During the first few days of an experiment, the dissolved oxygen concentration declines rapidly in an artificial stream receiving waste, as development of *Sphaerotilus* begins and carbohydrates present in the waste undergo increasing bacterial decomposition (Figure 7). When the *Sphaerotilus* growth is light, the transfer of surface water to the animal habitat beneath and among the large rocks of the bottom is not greatly obstructed and little difference exists between the dissolved oxygen concentrations in the water above the rocks and in the water below or between the rocks. Likewise, no considerable oxygen concentration differences between the water above the rocks and the water near the undersides of the large rocks of the control stream were observed. As can be seen in Figure 7, the concentration in the control stream is fairly constant. With the development of a medium to heavy benthic growth of *Sphaerotilus* in the stream receiving waste, the vertical exchange of

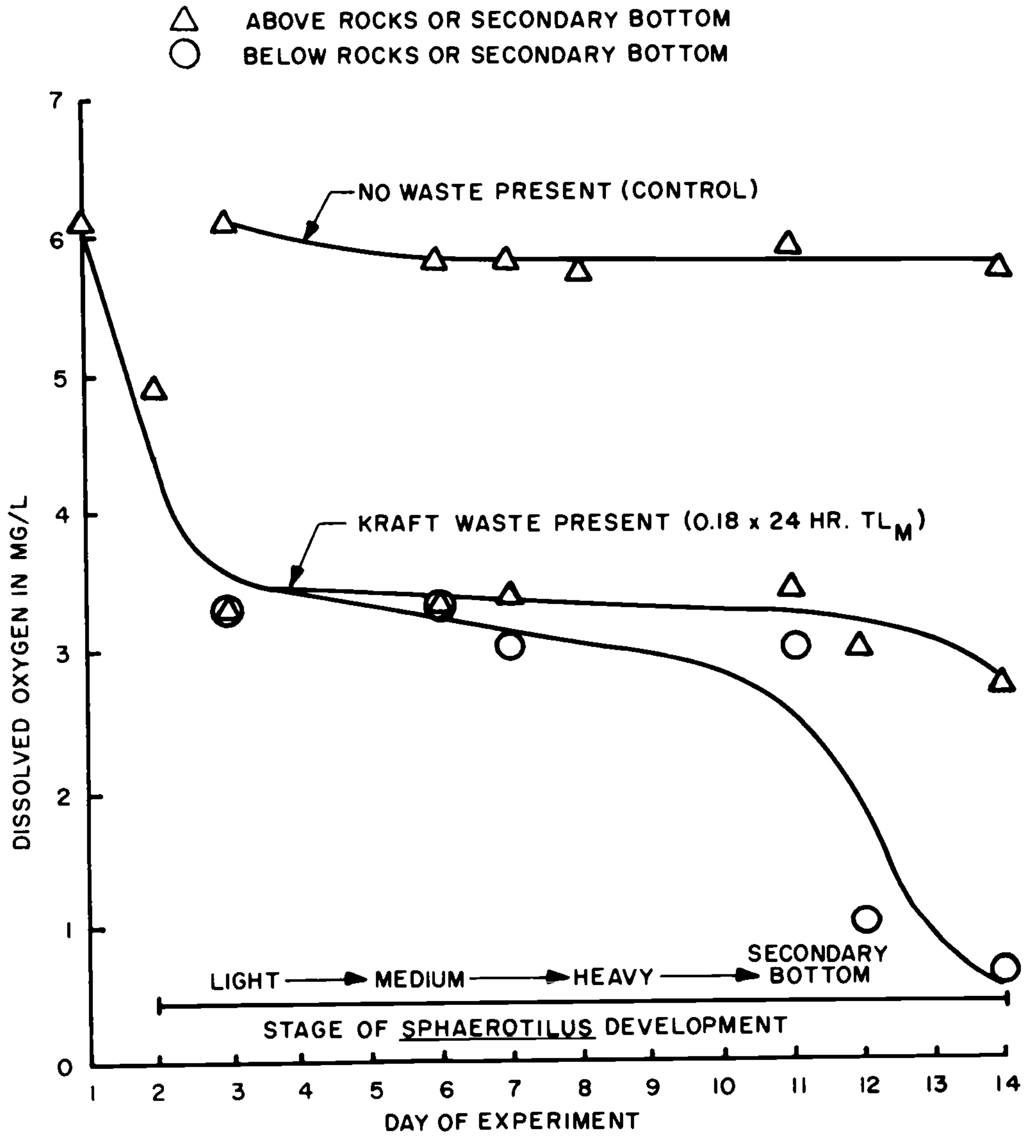

Figure 7. Showing the progressive development of dissolved oxygen and slime conditions in an artificial stream riffle receiving kraft waste. The oxygen conditions in a similar unpolluted stream are provided for comparison.

water begins to be obstructed and differences between the dissolved oxygen concentrations above and among the rocks become apparent. The development of a secondary bottom when growth conditions are suitable usually requires about 10 days; and once it exists, the vertical exchange of water becomes severely restricted, and the dissolved oxygen concentration below the secondary bottom declines rapidly and may soon approach zero. Once maximum development of Sphaerotilus for particular circumstances has taken place in an artificial stream, conditions tend to become stabilized whether or not a secondary bottom has developed. In sulfite waste experiments with artificial streams, light or medium growths of Sphaerotilus developed at the waste concentration of 300 mg/l; medium growths developed at concentrations of 600 and 800 mg/l; and medium to heavy growths or secondary bottoms developed at the concentration of 1,000 mg/l. Under natural stream conditions, the relationship between Sphaerotilus development and waste concentration would not necessarily be the same.

The concentration of dissolved oxygen in the artificial streams gradually declined over a period of several days after introduction of waste was begun. Also, the concentrations of oxygen throughout the experiments were highest near the surface and lower near the bottoms of the troughs, particularly when slime growths were well developed. Crayfish were always located on the bottom of the troughs. Snails and caddisfly larvae were frequently visible on top of the rocks, as well as on the bottoms of the troughs, and they may have tended to move upward from the bottom when oxygen concentrations were low. Stonefly naiads normally remained out of sight under the rocks, but at times of severe oxygen stress, some emerged from beneath the bottom materials and remained exposed to view on top of the rocks, on the sides of the troughs, or on the screens at either end of a trough. The variability in individual animal location, in addition to the temporal and vertical differences in the concentration of dissolved oxygen, made it uncertain to which oxygen concentrations the test results should be properly referred.

Of course, high concentrations of oxygen during the early days of an experiment would not offset the harmful effects of low concentrations that occurred and may have caused the death of animals which died later in an experiment. Therefore, the mean of all concentrations from the beginning to the end of an experiment would not very well represent the reduced concentration which may have resulted in deaths of test animals. The zone of water below the rocks in a stream is described as "dead" by Ambuhl (2, p. 142 and 175) even though there is considerable turbulence in the upper part of this zone. In this zone in the artificial streams, the lowest oxygen concentrations occurred at any given time, and it is in this zone that most test animals were located, even when oxygen concentrations were near or at lethal levels. Thus, all results of the stream experiments are referred to the lowest oxygen concentration which was recorded in each experiment. Once the growth of slime reached its maximum level of development, the concentrations of dissolved oxygen remained fairly constant during the remainder of an experiment. The lowest recorded concentration of oxygen was usually only very slightly lower than several concentrations recorded at other times toward the end of an experiment, indicating that the lowest recorded concentration reflected a general and sustained level of oxygen concentration.

The physical obstruction of the openings among the rocks of the bottom by bacterial slime not only may influence water quality and reduce water velocities and turbulence, but also in other ways may render the benthic environment unsuitable or less suitable for most riffle-dwelling animals. Further, Sphaerotilus natans can grow on the insect naiads or larvae, this having been observed at all sulfite waste concentrations tested. The growth of "sewage fungus" (bacteria and protozoa) on some aquatic insects that had been washed into the polluted section of a stream receiving domestic sewage has been observed by Tarzwell and Gaufin (46), who have attributed to it the death of these insects in the polluted water. One of the two *Acroneuria pacifica* naiads shown in Figure 8 was from an artificial stream receiving sulfite waste and has a rather dense growth of *Sphaerotilus* over the head, thorax, thoracic gills, legs, abdomen, anal gills, and caudal cerci. The delicate, filamentous tracheal gills on the thorax and in the anal region can be seen in the photograph of the naiad from the control stream. Obstruction of the flow of water over these gills and other respiratory surfaces probably constitutes a respiratory problem, particularly at oxygen levels which are critical even for normal animals. Further, by impeding the movements of the organisms, the *Sphaerotilus* may restrict their necessary activities and unduly increase the consumption of energy required for the activities. Some of the effects of *Sphaerotilus* growth on benthic animals and their environment have been considered; the possible effects of *Sphaerotilus* on the availability of plant food to benthic herbivores also must not be overlooked.

Figure 6 reveals that the variability of survival of *Acroneuria* sp. in artificial streams receiving sulfite waste at the various concentrations is high. The poor

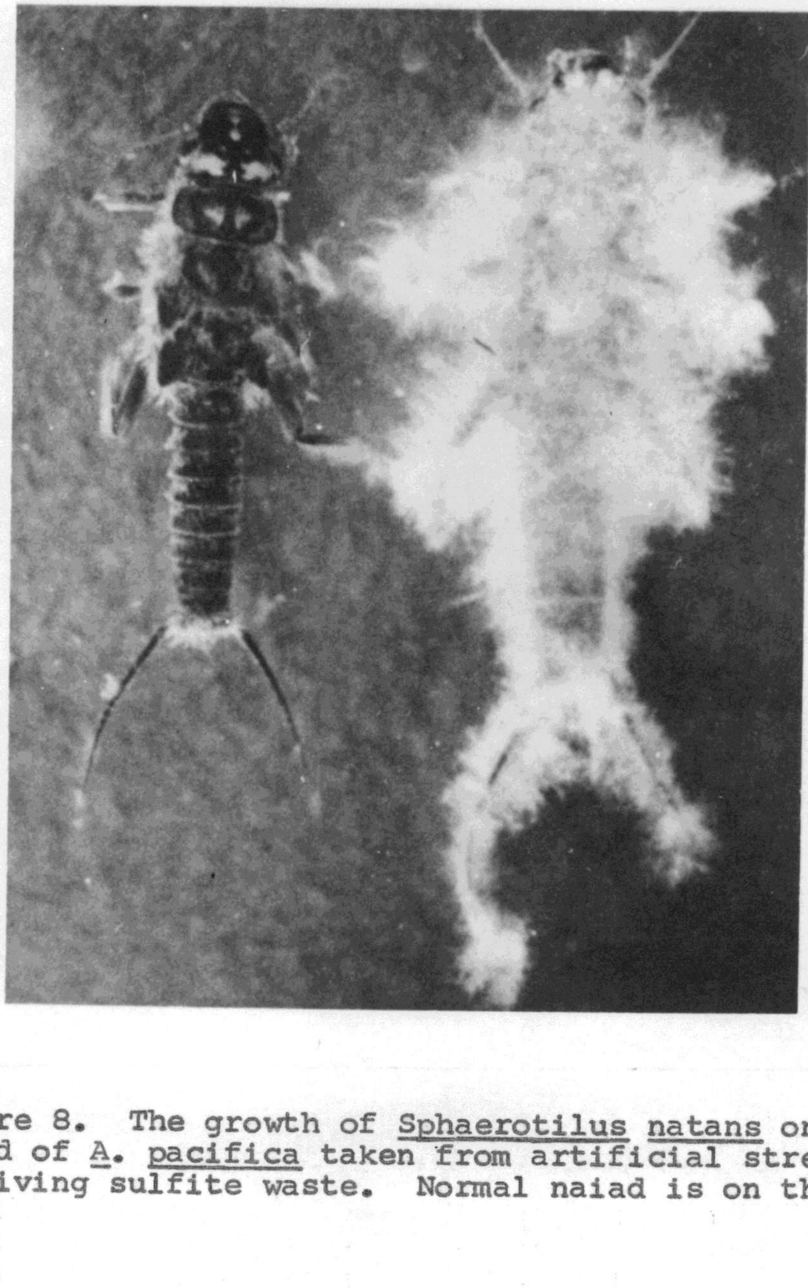

Figure 8. The growth of Sphaerotilus natans on a naiad of A. pacifica taken from artificial stream receiving sulfite waste. Normal naiad is on the left.

fit to the points of any curve that may be drawn suggests no simple relationship between waste concentration and per cent survival. This fit would have been made even poorer by inclusion of data not included in Figure 6 from experiments in which the dissolved oxygen concentration of water containing much waste was increased through the addition of oxygen to the incoming water. In these experiments, rather high survival percentages were obtained at waste concentrations of 800 and 1,000 mg/l when the lowest recorded dissolved oxygen concentration above or among the rocks was 4.0 mg/l or more. At a waste concentration of 800 mg/l, a 63 per cent survival was obtained when the minimum recorded dissolved oxygen concentration was 4.0 mg/l; while at the 1,000 mg/l waste concentration, a 65 per cent survival was obtained with a minimum recorded oxygen concentration of 5.6 mg/l. Figure 9, which includes data from experiments in which oxygen gas was introduced, shows the fairly good fit to the individual observations of a line expressing the relation between per cent survival and the lowest recorded dissolved oxygen concentration when sulfite waste was present. While such a fit alone is certainly not evidence that low dissolved oxygen concentration was the primary cause of high mortality rates, when considered together with the very poor fit to the individual observations of any curve expressing the relationship between per cent survival and waste concentration, it is a reason for believing that dissolved oxygen deficiency may well have been the chief cause of death.

Further evidence that dissolved oxygen deficiency is the primary cause of increased mortality is obtained when the percentages of survival of Acroneuria in all sulfite waste experiments in which the minimum recorded dissolved oxygen concentration was not below 4.0 mg/l are considered. Figure 10 includes the results of experiments in which the minimum dissolved oxygen concentration either above or near the undersides of the large rocks was never observed to be less than 4.0 mg/l. It will be noted that the position of a curve expressing the relationship between per cent survival and sulfite waste concentration up to 1,000 mg/l is significantly above that of the curve in Figure 6 from which the data from experiments having low minimum oxygen concentrations were not eliminated. The fact that Figure 10 suggests survival to be less in the presence of sulfite waste even when the recorded minimum dissolved oxygen concentration is 4.0 mg/l or more may have several explanations. First, experiments in which sulfite waste was present were more likely than control experiments to have had dissolved

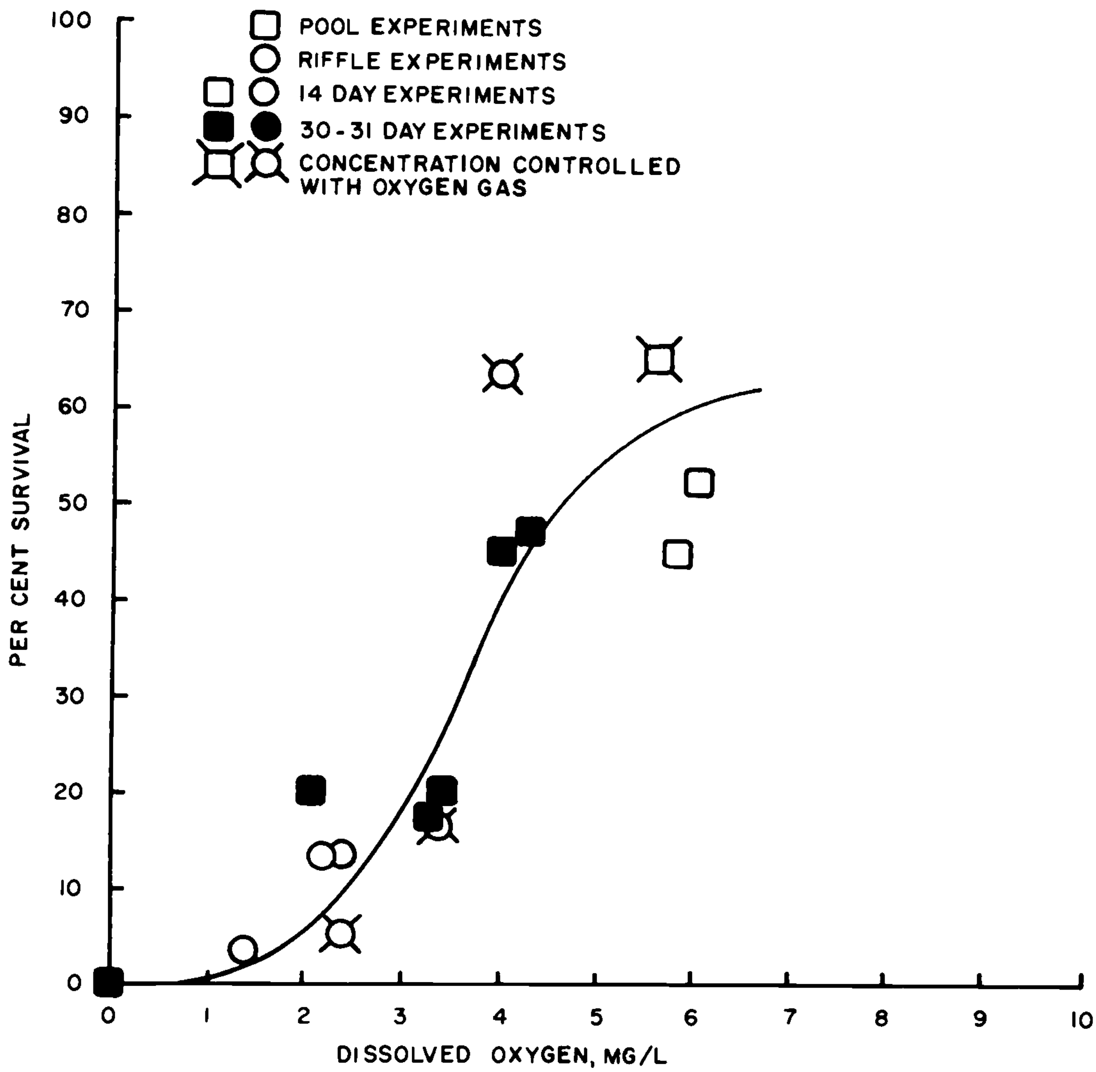

Figure 9. Survival of Acroneuria in relation to lowest recorded oxygen concentrations in sulfite waste experiments.

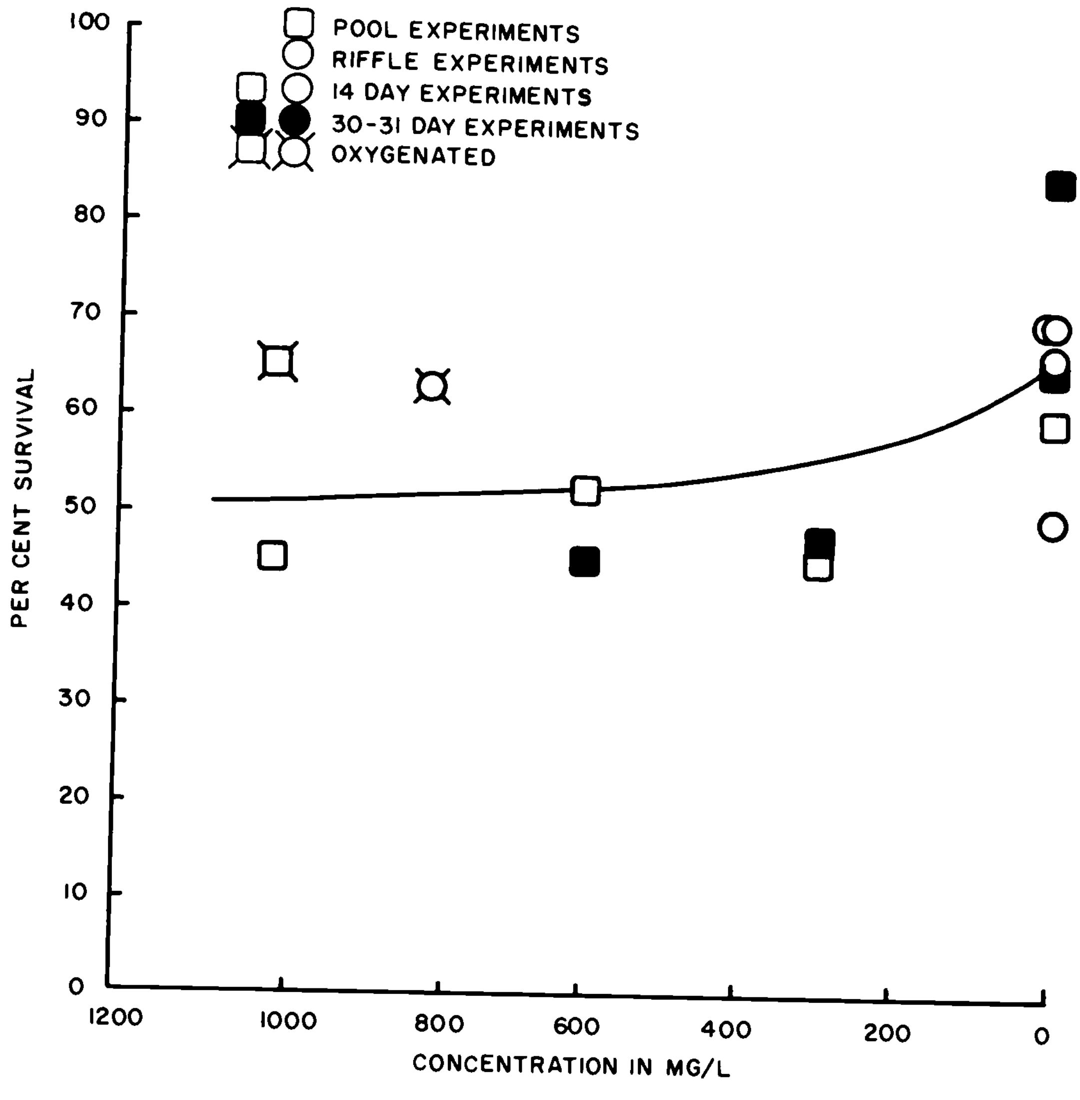

Figure 10. Survival of Acroneuria in relation to the concentration of sulfite waste liquor in experiments in which the lowest recorded oxygen concentration was 4.0 mg/l or greater.

oxygen minima below 4.0 mg/l which were not observed and which, had they been noted, would have been reason for elimination of the test results from the figure. Secondly, survival of Acroneuria tends to be greater at concentrations above 4.0 mg/l than at this concentration (Figures 9 and 18). Finally, survival may be influenced to some extent by factors other than dissolved oxygen which result in some degradation of the environment even while the dissolved oxygen remains adequate, or cause an increase of the dissolved oxygen requirement of the insects.

Considerable mortality of Acroneuria in the process of ecdysis was observed when survival was low in artificial stream experiments with sulfite waste. This is not a surprising observation, as organisms in different stages of development often differ in their tolerance to adverse environmental conditions.

In artificial streams in which large amounts of Sphaerotilus developed and in which dissolved oxygen concentrations were low, Acroneuria naiads tended to move upward from beneath the rocks on the bottom to the upper surfaces, where they were exposed to water currents of greater velocity. A few measurements of current velocity in the absence of Sphaerotilus were made using a Leupold-Stevens "Midget Current Meter." Representative velocities in one trough were 0.42 f.p.s. on the surface, 0.31 f.p.s. just above the large rocks, 0.15 f.p.s. between the large rocks, and less than 0.1 f.p.s. below the layer of large rocks. Large quantities of *Sphaerotilus* would no doubt considerably reduce the between-rock and under-rock velocities, and as water velocity is very important in determining the dissolved oxygen available to animals, the effects on the animals of oxygen deficiency are compounded. While the behavior of the naiads may have favored their survival under some adverse conditions in the artificial streams, in a natural environment it could render them much more susceptible to predation by fish and other animals.

The large, herbivorous stonefly naiads of *Pteronarcys* sp. did not appear to differ greatly from the *Acroneuria* naiads in their tolerance of conditions in the artificial streams resulting from the introduction of sulfite waste. Figure 11 shows the relationship between lowest recorded dissolved oxygen concentrations and survival of *Pteronarcys*. High survival percentages were not unusual with *Pteronarcys*. The usually high mortality of *Acroneuria* in control streams was probably due largely to cannibalism and to the predation of one species of *Acroneuria* on the other. The

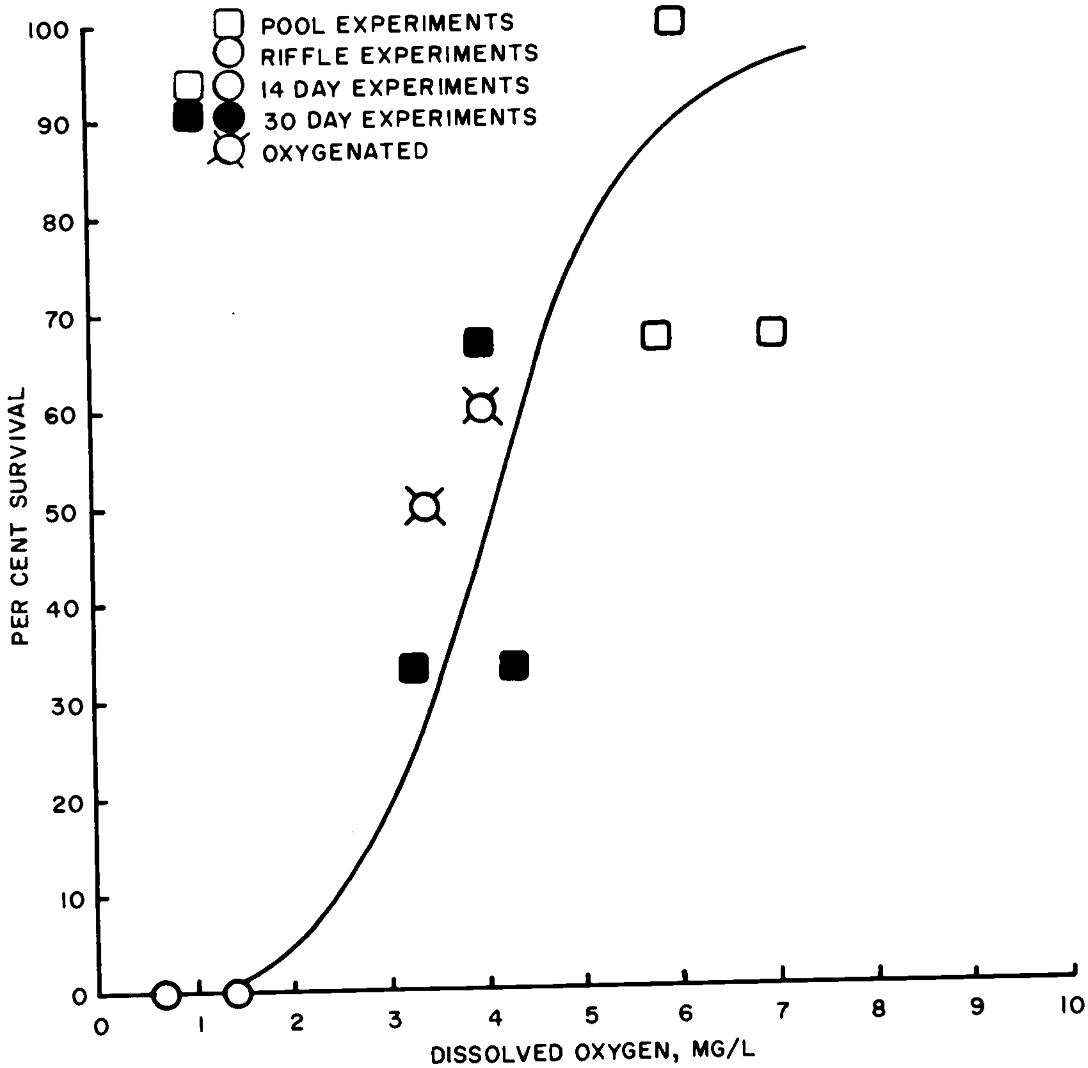

Figure 11. Survival of Pteronarcys in relation to lowest recorded concentrations of dissolved oxygen in experiments with sulfite waste.

large size of Pteronarcys may have prevented predation (by Acroneuria) and its herbivorous nature precluded cannibalism. It appears that there were greater losses of Acroneuria due to cannibalism and predation when the concentration of dissolved oxygen was more or less adequate, i.e., when there was little or no physiological stress due to low oxygen.

The emergence of some Acroneuria occurred in sulfite waste experiment No. IV. Both Acroneuria and Pteronarcys emerged in a preliminary experiment in which no waste was added to the water. In the sulfite waste experiment there was not entirely adequate provision made for the capture and enumeration of the emerged insects, and the recorded survival of naiads plus emergents was no doubt lower than the actual survival. As there was no emergence of Acroneuria, Pteronarcys, or limnephilids in any other experiment with sulfite waste, losses of these animals in the other experiments cannot be attributed to emergence.

No test animals were known to have crawled completely out of the troughs during any of the experiments. The continuous illumination of the troughs may have served as an effective deterrent to the crawling out of immature aquatic insects such as Acroneuria, which normally live well out of direct light and which could have easily left the troughs by crawling. In experiments with naiads of Acroneuria pacifica in troughs similar to those used in the present study, but not illuminated at night, Gerald E. Davis of Oregon State University often found live naiads on the floor beneath the troughs in the morning.

As in the case of Acroneuria, the dubious correlation between survival of Pteronarcys and the concentration of sulfite waste liquor when the concentration of dissolved oxygen was not less than 4.0 mg/l (see Figure 12) is evidence that insufficient dissolved oxygen is a major factor in the mortality of these animals.

Results obtained with large, herbivorous, pebble-cased caddisfly larvae of the family Limnephilidae (Figure 13) indicated that these insects did not differ greatly from the Acroneuria in their tolerance of conditions of low dissolved oxygen resulting from the presence of sulfite waste. Case-caddisfly larvae of the species used, like Pteronarcys naiads, are not cannibalistic and are either large enough or well enough protected so that deaths among these insects in the stream experiments cannot be attributed to cannibalism or predation. A 75 per cent survival of the caddisfly larvae, which was not much less than that of controls, was obtained at a sulfite

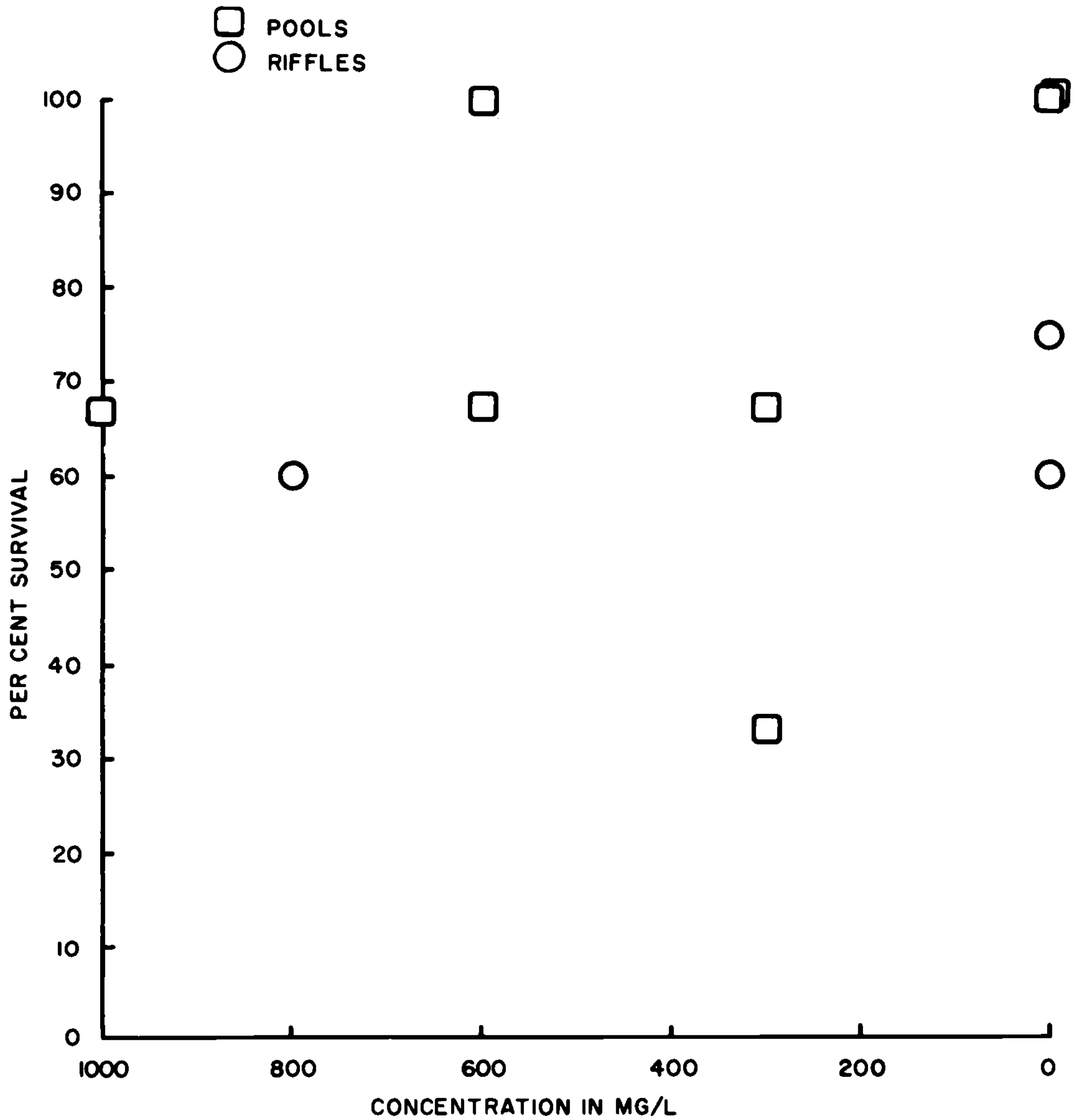

Figure 12. Survival of Pteronarcys and concentration of sulfite waste liquor at recorded oxygen concentrations not below 4.0 mg/l.

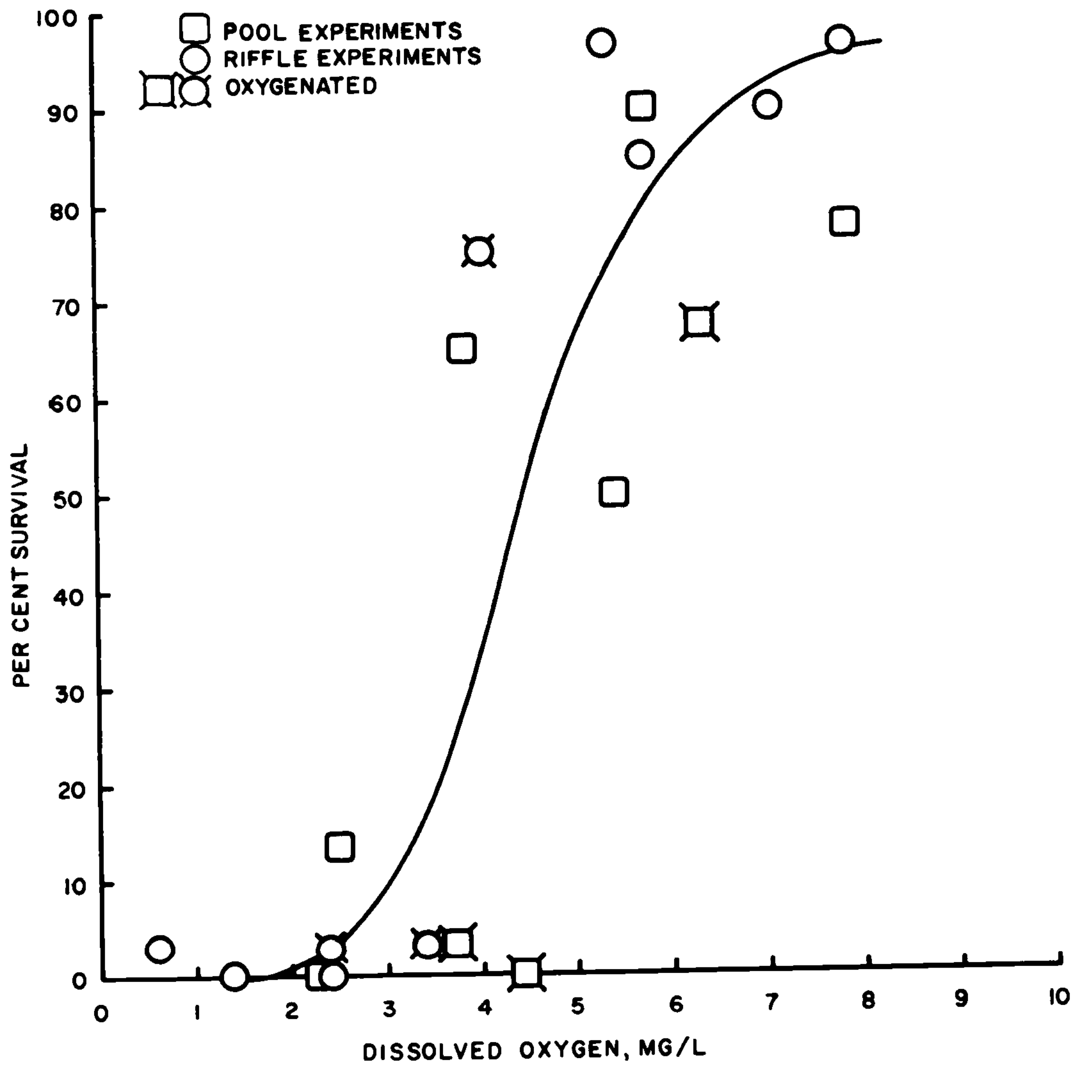

Figure 13. Survival of limnephilid larvae (from Alsea River) in relation to lowest recorded dissolved oxygen concentrations in 14 day experiments with sulfite waste.

waste concentration of 800 mg/l in an experiment in which oxygen was added to the water and the minimum recorded dissolved oxygen concentration was 4.0 mg/l. The upward adjustment of dissolved oxygen in streams receiving sulfite waste always increased the survival of stonefly naiads, caddisfly larvae, and crayfish (Table 6). This once more suggests that oxygen deficiency is an important factor contributing to the death of invertebrates at high sulfite waste concentrations. Admittedly, not all the evidence points unequivocally to oxygen deficiency as the dominant lethal factor, for unexpectedly poor survival was observed of all species of test animals in experiment No. VI and of crayfish in experiment No. V in troughs in which the lowest recorded dissolved oxygen concentrations were not very low. However, the unusually high temperatures prevailing during these experiments may have been responsible for these results.

The percentage of mayflies of the genus Isonychia successfully emerging in a pool experiment was lower at the concentrations of sulfite waste tested than in the control test. The artificial streams having been equipped for collecting the emerged mayfly subimagos, it was found that, out of 13 individuals placed in each stream, 11 emerged from the control stream, 6 from the stream with a waste concentration of 300 mg/l, and 2 each from the

Table 6. Comparison of Survival of Various Test Animals With and Without Oxygen Adjustment (Upward) in Simultaneous Tests with Sulfite Waste Liquor Concentrations of 1000 mg/l or less.

Exper. No.	Test Animal	No Adjustment		Adjustment		Waste Liquor Concentration mg/l
		Per Cent Survival	Min. O_2, mg/l	Per Cent Survival	Min. O_2, mg/l	
IV	*Acroneuria* naiads	25.0	5.2	65.0	5.6	1000
	Limnephilid larvae	10.0	5.2	60.0	5.6	do
V	*Acroneuria* naiads	13.3	0.6	63.3	4.0	800
	Pteronarcys naiads	0.0	0.6	60.0	4.0	do
	Limnephilid larvae	3.3	0.6	75.0	4.0	do
	Pacifastacus trowbridgi	0.0	0.6	20.0	4.0	do
VI	*Acroneuria* naiads	3.3	1.4	16.7	3.4	800-1000[1]
	Limnephilid larvae	0.0	1.4	3.3	3.4	do
	Pacifastacus trowbridgi	0.0	1.4	18.8	3.4	do

1 Waste concentration of unadjusted test was 800 mg/l; 1000 mg/l in the test with adjusted oxygen.

streams with waste concentrations of 600 and 1,000 mg/l. At the conclusion of the experiment, one naiad remained alive in the control stream, the other having succumbed for some unknown reason. Of the mayflies failing to emerge from the streams receiving waste, all died during the process of emergence or without having crawled out of water to emerge.

In another (14 day) experiment with _Acroneuria pacifica_ and _Acroneuria californica_ at concentrations of 300 and 1,000 mg/l of waste, many of the naiads were able to emerge successfully as adults and there was even reason to believe that the waste somehow caused earlier emergence. However, because of the artificial adjustment upward of the oxygen content in the trough containing 1,000 mg/l of waste, the lowest recorded dissolved oxygen concentration, unlike that in the experiment with _Isonychia_, never dropped below 5.4 mg/l.

There was evidence that some concentrations of sulfite waste favored the establishment of some species of chironomids (Tendipedidae). Although they were not placed in known numbers into the artificial streams, the chironomids under some conditions became established in the streams, evidently having been introduced through the water supply. In spring and fall experiments, the chironomids became abundant and emerged from the streams having waste concentrations from 300 to 1,000 mg/l, while they were apparently absent or rare in the control streams. The increased amount of detritus and other materials that accumulated in the troughs receiving the waste and the possible use of bacterial growths as food may have been factors favoring the establishment of chironomids.

Very high percentages of survival of the crayfish, *Pacifastacus trowbridgi*, were obtained at sulfite waste concentrations of 300, 600, and 1,000 mg/l in experiments where conditions of temperature and dissolved oxygen were favorable. Survival percentages comparable to those of controls, which were always high, also were obtained with the gastropods *Fluminicola* and *Goniobasis* at a waste concentration of 1,000 mg/l.

The cladoceran, *Eurycerus lamellatus*, became established during an artificial stream pool experiment with sulfite waste. This organism became about twice as abundant in the stream receiving 300 mg/l sulfite waste as in the control stream. At the waste concentration of 600 mg/l it was about five times as abundant, and at the concentration of 1,000 mg/l it was about ten times as abundant as in the control stream.

While a varied flora of at least 46 species of diatoms and other algae occurred in the benthic environment of two control streams in one experiment, only small numbers of individuals of two or three species were observed in streams having sulfite waste concentrations of 600, 800, and 1,000 mg/l. This depression of plant production (other than bacterial production) contrasted rather strikingly with the stimulation of chironomid and cladoceran production noted above.

No deaths of young steelhead trout, *Salmo gairdneri gairdneri*, occurred at sulfite waste concentrations of 300 and 600 mg/l in one 27-day experiment in which temperatures ranged from 13 to 21°C., though there was a 30 per cent mortality at the concentration of 1,000 mg/l. The steelhead trout held at waste concentrations of 300 and 600 mg/l showed weight gains of 35.8 and 13.8 per cent respectively; while the gain in weight of the controls was 45.2 per cent. These fish were fed a prepared diet of beef liver, fish, and supplements. The apparent influence of sulfite waste on fish growth could be due to some toxic action of the waste or due to lowered oxygen concentration, the latter having been shown to adversely affect the growth of salmonids (Herrmann, Warren, and Doudoroff, 26, p. 157-167). The mean dissolved oxygen concentrations in the control stream and in streams with waste concentrations of 300 and 600 mg/l were 6.9, 5.3, and 5.2 mg/l respectively. The last two concentrations do not appear to be low enough or different enough from that in the control stream to account entirely for the observed differences of the weight gains of the trout, and furthermore, they may be lower than the true concentrations because of interference with the modified Winkler test which was employed. Mortalities of steelhead trout in 14 days in a second experiment having the much lower temperature range of 4 to 13°C. were 10, 55, and 80 per cent at waste concentrations of 300, 600, and 1,000 mg/l, respectively. There were no deaths among the control fish. The possible influence of very low temperatures or other seasonal factors on the lethal concentrations of sulfite waste suggested by these two experiments should be investigated.

No deaths of chinook salmon occurred in 20 days at temperatures ranging from 8 to 16°C. in artificial streams receiving 300 and 600 mg/l sulfite waste. A mortality of 60 per cent was reached on the 8th day at the concentration of 1,000 mg/l, no further deaths occurring through the 20th day. In a second experiment in which temperatures ranged from 13 to 19°C., no deaths occurred at the concentration of 300 mg/l, whereas at the concentration of 1,000 mg/l, 50 per cent mortality was reached on the 7th day, and there were no further deaths through the 12th day.

In none of the tests with fish was the death of the animals attributable to reduced oxygen concentration. Chronic toxicity of sulfite waste liquor to juvenile chinook salmon, resulting in the death of some of the fish after prolonged exposure to liquor concentrations between 600 and 1,000 mg/l, has been reported by Williams, *et al.* (54, p. 35-44).

Experiments with kraft waste liquor

Artificial stream experiments were performed to determine some of the effects on various aquatic organisms of concentrations of kraft waste ranging from 0.01 to 0.3 times the 24-hour median tolerance limit for guppies. The 24-hour TL_m for guppies of the samples of kraft waste used ranged from 1.7 to 8.7 per cent by volume. Tables 7 and 8 summarize some salient conditions and results of these experiments. In order to maintain the desired toxicity levels in the different experiments, the prepared kraft wastes used were introduced in amounts sufficient to produce concentrations ranging from 0.024 to 1.6 per cent by volume. As indicated earlier, the kraft waste used was prepared with combined condensates and black liquor and no additional water, and so it was more concentrated than most kraft pulp and paper mill effluents.

The problem of determining which of the conditions

Table 7. Summary of Conditions and Some Results of Experiments with Kraft Liquor.

Exper.	Incl. Dates	Length in Days	Type	Bottom Type	Water Inflow ml/min	Surf. Veloc. ft/sec	Liquor Concen.[1]	Temp. Range °C.	Recorded Range of Dissolved Oxygen[2] Above Rocks Min. mg/l	Above Rocks Max. mg/l	Under Rocks Min. mg/l	Under Rocks Max. mg/l	Slime Develop.	Slime and Debris[3] c.c.	Bioassay Animal
I	July 29-	(35)	pool	1	1400	nil	0.30	17.8-25.6	3.0	5.7	-	-	sec. bot.	7000	sculpin
	Sept. 2,		do	do	do	do	0.10	do	6.0	6.4	-	-	-	3640	do
	1955		do	do	do	do	0.01	do	6.9	7.2	-	-	-	2160	do
			do	do	do	do	control	do	7.1	7.3	-	-	-	1740	-
Special A	Sept. 29-	(11)	riffle	1	1000	0.4	0.30	13.9-21.7	-	(4.0)[5]	-	-	sec. bot.	-	guppy
	Oct. 9,		do	do	do	do	control	do	6.0	6.6	-	-	none	-	-
	1956														
Special B	Oct. 31-	(65)	riffle	1	1000	0.4	0.50	15.0-18.4	-	(3.3)[5]	(-)[4]	-	sec. bot.	-	do
	Jan. 4,		do	do	do	do	control	do	-	(6.6)[5]	-	-	none	-	-
	1956														
II	Oct. 14-	(35)	riffle	2	1000	0.4	0.20	15.0-19.5	3.9	5.6	0.4	4.0	sec. bot	10300	do
	Nov. 18,		do	do	do	do	0.06	15.0-22.5	5.5	6.0	5.2	5.4	light	3700	do
	1956		do	do	do	do	0.02	15.0-20.0	6.0	6.5	6.0	6.4	light	2300	do
			do	do	do	do	control	do	6.2	6.8	-	-	none	1600	-
III	Feb. 16-	(24)	riffle	2	1000	0.4	0.20	11.7-18.9	4.6	5.7	2.8	4.4	sec. bot.	7700	do
	Mar. 12,		do	do	do	0.5	0.10	11.7-16.7	6.3	8.5	(8.3)[5]	-	medium	8500	do
	1957		do	do	do	0.4	0.05	13.4-18.4	6.2	6.7	(6.0)[5]	-	light	3800	do
			do	do	do	0.5	0.025	do	(7.3)[5]	-	(6.3)[5]	-	light	3900	do
			do	do	do	0.3	control	13.4-18.9	-	(6.4)[5]	(6.8)[5]	-	none	4600	-
			do	do	do	0.5	control	do	-	(7.2)[5]	(6.8)[5]	-	none	3400	-
IV	June 22-	(14)	riffle	2	1500	0.4	(0.18)[6]	22.2-24.5	4.8	8.0	4.9	7.0	heavy	7000	guppy
	July 5,		do	do	do	do	0.18	do	2.7	4.9	0.6	3.3	sec. bot.	6000	do
	1957		do	do	do	do	control	do	5.7	5.9	-	-	none	450	-
			do	do	do	do	control	do	7.0	8.2	-	-	none	400	-
			pool	no rocks	do	nil	(0.18)[6]	22.2-25.0	6.3	6.9	-	-	-	-	guppy
			do	do	do	do	0.18	22.2-24.3	3.8	4.2	-	-	-	-	do

1 Fraction of 24-hr. TL_m for guppy or sculpin (see remarks column).
2 See Footnote 1, Table 4.
3 See Footnote 2, Table 4.
4 Lowest oxygen concentration under secondary bottom was probably zero.
5 Only one determination was made.
6 River water oxygenated before entering trough.

Table 8. Survival of Test Animals in All Experiments with Kraft Liquor.

Exper. No.	Length in Days	Test Animal	0.3 TL_m			0.2 TL_m			0.18 TL_m			0.10 TL_m			0.06 TL_m			0.05 TL_m			0.025 TL_m			0.02 TL_m			0.01 TL_m			Control A			Control B		
			In	Out	%	In	Out	%	In	Out	%	In	Out	%	In	Out	%	In	Out	%	In	Out	%	In	Out	%	In	Out	%	In	Out	%	In	Out	%
I	(35)	Limnephilid larvae	128	0	0.0	-	-	-	-	-	-	64	0	0.0	-	-	-	-	-	-	-	-	-	-	-	-	64	63	98.4	64	62	96.9	-	-	-
Special A	(11)	A. pacifica and A. californica	20	0	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20	16	80.0	-	-	-
Special B	(65)[1]	do	10	0	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	8	80.0	-	-	-
II	(35)	A. pacifica and A. californica	-	-	-	15	0	0.0	-	-	-	-	-	-	15	9	60.0	-	-	-	-	-	-	15	9	60.0	-	-	-	15	8	53.3	-	-	-
		Pteronarcys sp.	-	-	-	6	0	0.0	-	-	-	-	-	-	6	5	83.3	-	-	-	-	-	-	5	4	80.0	-	-	-	5	5	100.0	-	-	-
		Goniobasis sp.	-	-	-	20	17	85.0	-	-	-	-	-	-	20	18	90.0	-	-	-	-	-	-	20	18	90.0	-	-	-	20	20	100.0	-	-	-
III	(24)	A. pacifica and A. californica	-	-	-	30	1	3.3	-	-	-	30	21	70.0	-	-	-	30	14	46.7	30	19	63.3	-	-	-	-	-	-	30	16	53.3	30	18	60.0
	(18)[2]	do	-	-	-	20	2	10.0	-	-	-	10	5	60.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	(24)	Pteronarcys sp.	-	-	-	8	1	12.5	-	-	-	8	4	60.0	-	-	-	8	6	75.0	8	2	25.0	-	-	-	-	-	-	8	4	50.0	8	4	50.0
		Hydropsychid larvae	-	-	-	10	1	10.0	-	-	-	10	7	70.0	-	-	-	10	5	50.0	10	5	50.0	-	-	-	-	-	-	10	5	50.0	10	4	40.0
IV	(14)	A. pacifica and A. californica	-	-	-	-	-	-	30	3	(10.0)[1]		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		do	-	-	-	-	-	-	30	0	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	15	50.0	30	20	66.7
		Pteronarcys sp.	-	-	-	-	-	-	5	4	(80.0)[1]		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		do	-	-	-	-	-	-	5	0	0.0		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	4	80.0	4	3	75.0
		Limnephilid larvae	-	-	-	-	-	-	60	10	(16.7)[1]		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		do	-	-	-	-	-	-	60	0	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	60	51	85.0	60	48	80.0
		Pacifastacus trowbridgi	-	-	-	-	-	-	8	1	(12.5)[1]		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		do	-	-	-	-	-	-	40	17	(42.5)[1]		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		do	-	-	-	-	-	-	40	0	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

1 Test animals subjected to 0.5 TL_m for 13 days in stainless steel screen capsule in artificial stream.
2 Special tests run in stainless steel screen capsules in artificial stream.
3 River water oxygenated before entering trough.

resulting from the presence of waste materials are primarily responsible for the destruction of a particular species is as difficult with kraft waste as with sulfite waste. With kraft waste, as with sulfite waste, low concentrations of dissolved oxygen, toxicity, and the blanketing of the benthic environment by Sphaerotilus may directly or indirectly bring about the death of different aquatic organisms. The relationship shown in Figure 14 between the per cent survival of Acroneuria naiads and the concentration of kraft waste (adjusted on the basis of acute toxicity to fish) indicates only that some factor or factors related to waste concentration (or to the acute toxicity) resulted in an increase of mortality at tested concentrations above 0.1 times the 24-hour TL_m for guppies. Alone, such relationship fails to provide a means for distinguishing between the injurious effects of toxicity, low concentrations of dissolved oxygen, the growth of Sphaerotilus, and other possible factors.

It was possible in the sulfite waste experiments to distinguish to some extent between the effects of low dissolved oxygen concentration and possible toxic or other effects of the waste on Acroneuria naiads by artificially maintaining the dissolved oxygen concentration at a relatively high level even with high waste

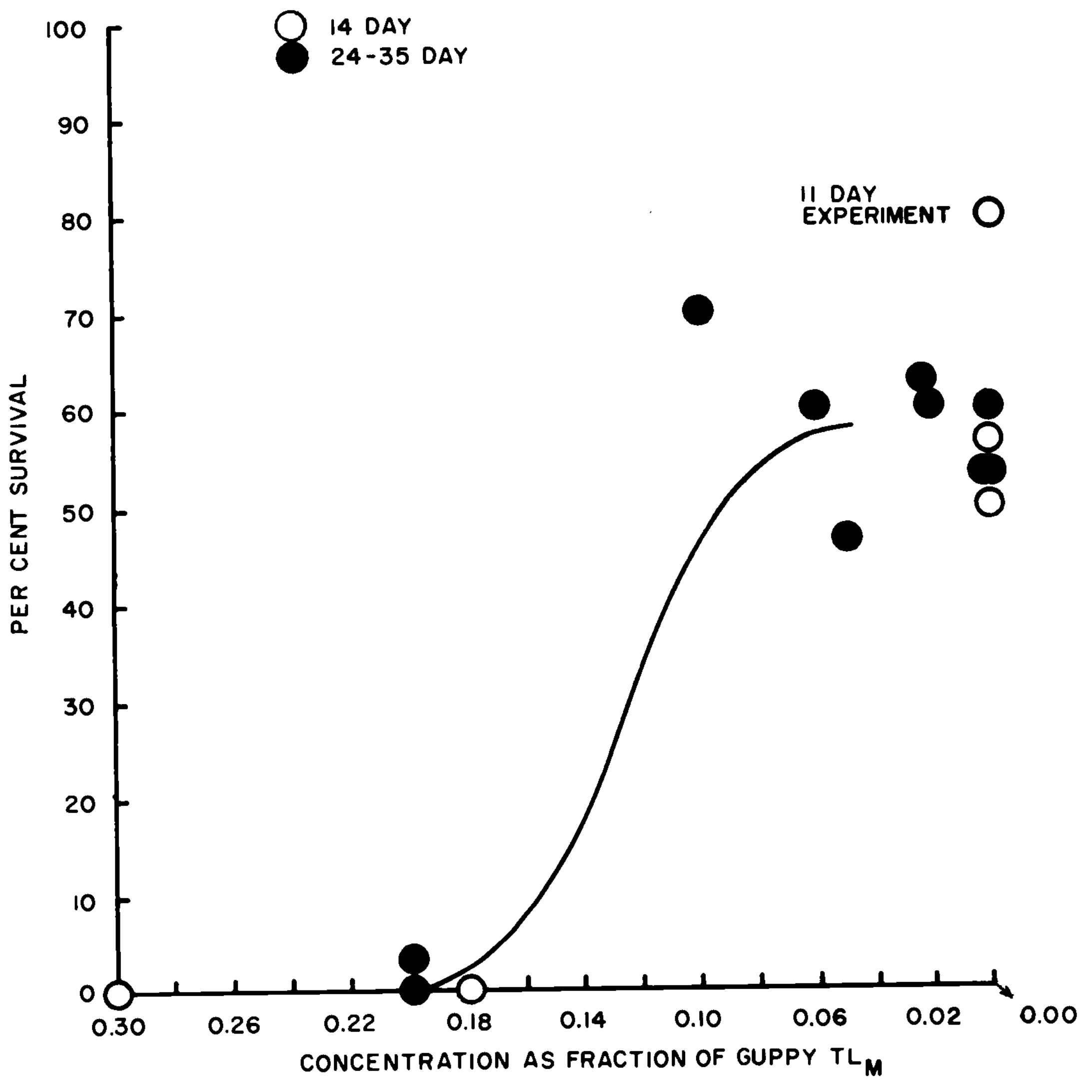

Figure 14. Survival of Acroneuria in relation to toxicity-adjusted concentrations of kraft waste in riffle experiments.

concentrations, and thus obtaining survival percentages nearly equal to those of controls. This was one indication that low dissolved oxygen might be the cause of death in the presence of sulfite waste. Only one experiment with kraft waste was performed in which oxygen was added to the incoming water in order to maintain a relatively high concentration of dissolved oxygen even at a high waste concentration. While certainly not conclusive evidence, the result of this one experiment suggested toxicity of kraft waste to be an important cause of death of *Acroneuria* naiads. At a concentration of 0.18 times the 24-hour TL_m for guppies, only 10 per cent survival of *Acroneuria* was obtained, even though the minimum recorded concentration of dissolved oxygen was 4.8 mg/l.

Correspondingly, only a 10 per cent survival was recorded in another experiment at a waste concentration of 0.2 times the 24-hour TL_m for guppies when the *Acroneuria* naiads were held in a wire cage placed in an artificial stream above the rocks on the bottom where the minimum recorded oxygen concentration was 4.6 mg/l, although the recorded minimum among the rocks was 2.8 mg/l. In a parallel test at one-half the above concentration of kraft waste, a 50 per cent survival was obtained, with a minimum recorded oxygen concentration of 6.3 mg/l. The rather frequent occurrence of distressed and dead Acroneuria naiads early in kraft waste experiments at relatively high waste concentrations, before dissolved oxygen concentrations fell below 4.5 mg/l, was another indication that toxicity may be an important lethal factor. Such early distress and mortality were not observed in sulfite waste experiments.

Figure 15 shows the relationship between the per cent survival of Acroneuria and the lowest recorded dissolved oxygen concentration in riffle experiments with kraft waste. The relationship which is apparent does not prove that reduced dissolved oxygen is a primary or even an important cause of death in artificial stream experiments with kraft waste. Such a relationship could be simply a result of a high degree of negative correlation between the concentration of dissolved oxygen and kraft waste concentration, with which the factor primarily responsible for death also happens to have a high degree of correlation.

The growth of Sphaerotilus in the artificial streams has not yet been considered as a possible major direct cause of increased mortality of insects in the artificial streams receiving pulp mill wastes. An increase of insect mortality, with increasing waste concentration, which could not be ascribed largely to oxygen deficiency has been attributed chiefly to toxicity of the introduced waste.

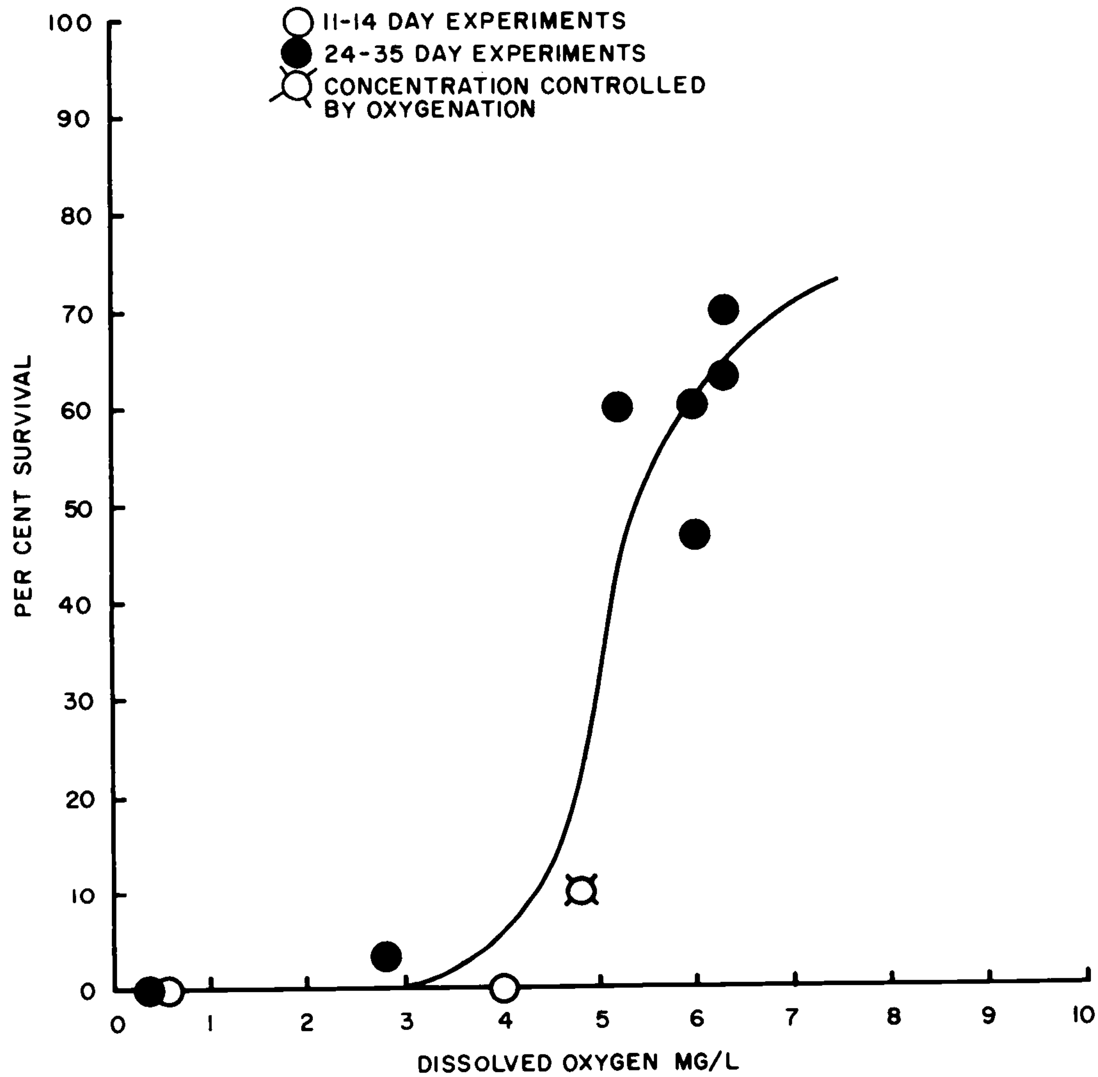

Figure 15. Survival of Acroneuria in relation to lowest recorded dissolved oxygen concentrations in riffle experiments with kraft waste.

The influence of Sphaerotilus growths on dissolved oxygen concentrations in the benthic environment already has been considered, and the relationship between Sphaerotilus development, oxygen concentration, and time, in an artificial stream experiment with kraft waste has been illustrated in Figure 7. If heavy growths of Sphaerotilus can markedly increase the mortality rate of insects such as Acroneuria in some way other than by promoting the reduction of dissolved oxygen and impeding the flow of water among the rocks of the stream bottom, this effect has not yet been demonstrated. Additional experiments need to be performed wherein growths of Sphaerotilus of varying density are produced while high oxygen concentrations are maintained and no possibly toxic substances are added to the water. For the present, it must be assumed that some of the high mortality of Acroneuria at rather high oxygen concentrations in artificial streams receiving kraft mill wastes was due partly to a direct lethal effect of the waste. The growth of Sphaerotilus upon aquatic insects and other organisms, described in connection with sulfite waste experiments, sometimes took place also in artificial streams receiving kraft waste, and must be considered as one of the possible inimical effects of the waste. However, Acroneuria which did not yet have any pronounced growth of Sphaerotilus upon them often were found dead or dying in the artificial streams, and for this reason the affliction in question is not deemed likely to have been the major cause of the observed mortality.

Generally, light growths of Sphaerotilus developed on the artificial stream bottoms at kraft waste concentrations as low as 0.02 times the 24-hour TL_m for the guppy. Medium growth occurred at a concentration of 0.1 times the 24-hour TL_m. Heavy growth of Sphaerotilus and the development of a secondary bottom generally occurred at a concentration of 0.18 times the 24 hour TL_m and at higher concentrations.

One experiment performed with a small number of Pteronarcys naiads yielded results that indicate that this form may not be as susceptible as Acroneuria naiads to the toxic action of kraft waste. When the concentration of dissolved oxygen was maintained at a high level by oxygenation, an 80 per cent survival of Pteronarcys was observed at a waste concentration of 0.18 times the 24-hour TL_m, whereas there was only a 10 per cent survival of the Acroneuria in the same experiment. With this notable exception, the results obtained with Pteronarcys in kraft waste experiments did not differ markedly from those obtained with Acroneuria (compare Figures 14 and 16, and 15 and 17). As in the sulfite

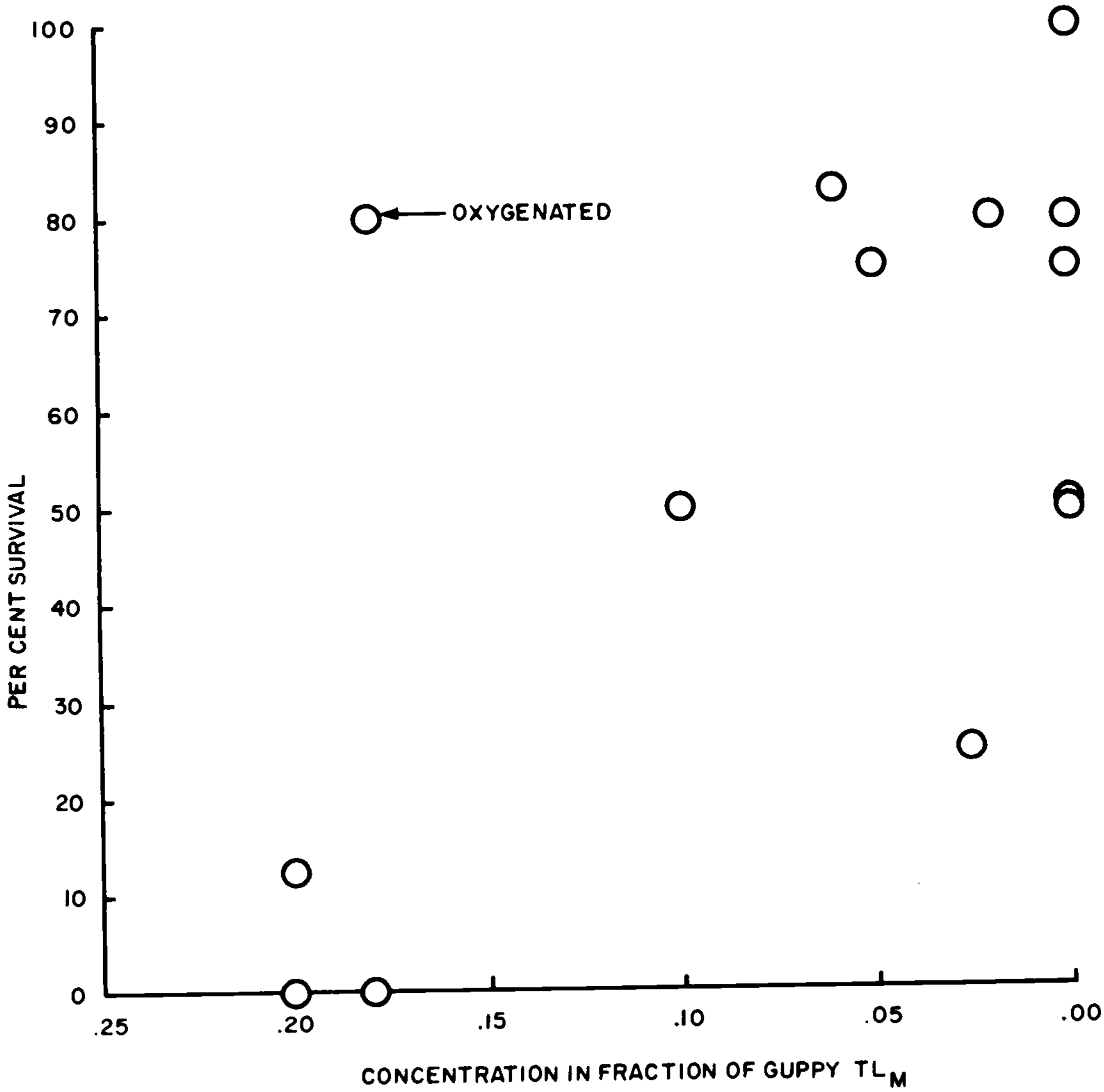

Figure 16. Survival of Pteronarcys in various dilutions of kraft waste in riffle experiments.

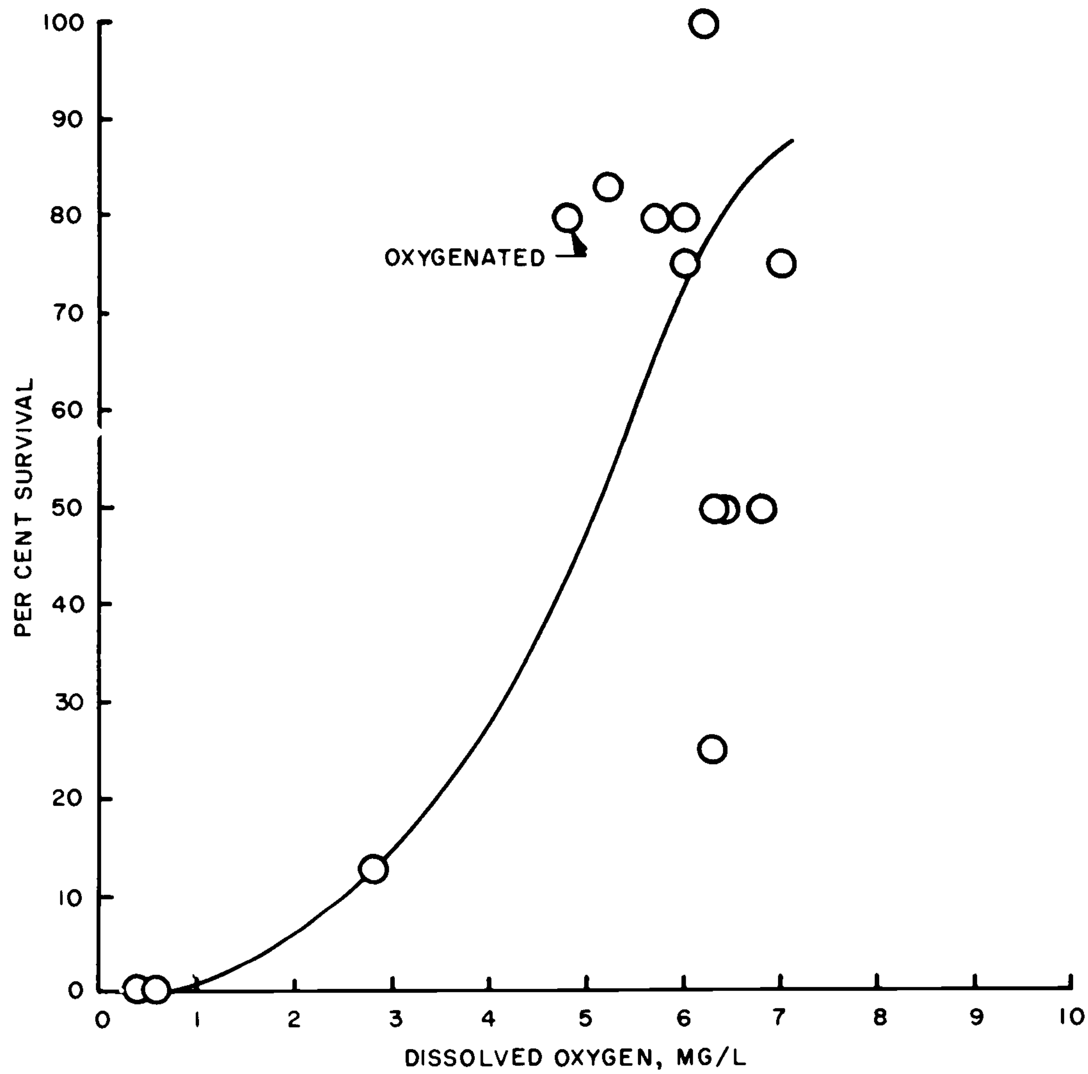

Figure 17. Survival of Pteronarcys in relation to lowest recorded concentrations of oxygen in riffle experiments with kraft waste.

waste experiments, generally higher control survival percentages were obtained with *Pteronarcys* than with *Acroneuria*. Similarly, high control survival percentages were again obtained with limnephilid caddisfly larvae. Limited work with the limnephilid larvae indicated their tolerance of conditions resulting from the introduction of kraft waste not to differ greatly from the tolerance shown by the *Acroneuria*. There were no known losses of the above forms due to emergence or crawling out of the troughs in any of the kraft waste experiments. Survival percentages of the gastropod *Goniobasis* in one experiment were 100, 90, 90, and 85 per cent at kraft waste concentrations of 0.0, 0.02, 0.06, and 0.2 times the 24-hour TL_m for guppies. Low oxygen concentrations occurred in this experiment among the rocks at the bottom of the trough with the highest waste concentration.

Chironomids did not become numerous in the benthic environment of the artificial streams during any of the kraft waste experiments. This is in striking contrast with the results of comparable sulfite waste experiments. Since conditions stimulating *Sphaerotilus* production apparently can favor some chironomids, the scarcity of chironomids in the kraft waste experiments even when *Sphaerotilus* was abundant suggests that this waste can, under some conditions, be inimical to the chironomids.

In natural streams receiving kraft wastes, however, large numbers of chironomids have at times been observed in the dense growths of benthic periphyton produced.

Of the 46 species of diatoms and other algae observed in the control streams mentioned above in connection with sulfite waste experiments, only one species was well represented in a simultaneous kraft waste test at a concentration of 0.18 times the 24-hour TL_m for guppies.

In two artificial stream experiments at a kraft waste concentration of 0.3 times the 24-hour TL_m for the sculpin Cottus perplexus, 50 per cent mortality of this same species occurred by the 7th and 9th days, the mortality being 100 per cent by the 9th and 11th days, respectively. Temperatures ranged from 18 to 25°C. At a waste concentration of 0.1 times the 24-hour TL_m, a mortality of 15 per cent was reached on the 32nd day, no deaths occurring at a concentration of 0.01 times the 24-hour TL_m, or among the controls. In these tests the lowest recorded oxygen concentration was 3.0 mg/l. Alderdice and Brett (1, p. 792) found that lowered dissolved oxygen increased the toxicity of kraft mill effluent to young salmon in bottles of sea water. At an effluent dilution of 4 per cent, hypoxial reactions were initiated by the fish when the dissolved oxygen content was lowered to 3.59 mg/l. At a dilution of 18 per cent, a reduction of the oxygen content to only 6.00 mg/l was required to produce the same reactions. Even though waste concentrations in the artificial streams were never as high as 4 per cent, the waste added to the streams was probably stronger than the effluent tested by Alderdice and Brett, thus, this interaction may have been a factor in the mortality of the sculpins in the streams.

It should be noted that any toxicity to both fish and insects of diluted kraft waste to which the animals were exposed in artificial streams may have been significantly less than the toxicity of comparable fresh dilutions of the waste. Some loss of toxicity evidently occurred during the period of retention of the waste dilutions in the artificial streams in contact with rocks overgrown with bacterial periphyton. In a single preliminary test, all of a group of chinook salmon soon succumbed in a trough receiving a rather concentrated, untreated dilution (0.5 x 24-hour TL_m for guppies). They died before any mortality occurred among steelhead held in a like trough ("pool") which received waste of the same concentration overflowing from the "riffle" of an artificial stream, where the diluted waste was being circulated over rocks with a lush bacterial growth (retention time about one hour). There is other evidence that the toxicity of kraft mill wastes can be rapidly reduced by bacterial action under certain favorable conditions.

Experiments with unpolluted water

In order to obtain general information on the low oxygen tolerance of *Acroneuria* in the absence of waste, 14-day artificial stream experiments were performed in which the concentration of dissolved oxygen was controlled by bubbling either nitrogen or oxygen through the incoming river water. In these experiments the general physical conditions were the same as in the prior experiments with sulfite and kraft wastes in the riffle and pool apparatus. Tables 9 and 10 summarize the conditions and results of these experiments. The naiads of *A. californica* and *A. pacifica* were enumerated separately, although the results are combined at times for comparison with the results of earlier pulp mill waste experiments in which this separation was not made.

Lowest recorded oxygen concentrations in riffle and pool troughs were plotted against survival of the two species in Figures 18, 19, and 20. Figure 20 includes data not shown in Figure 18 on *A. pacifica* held in screen capsules in the troughs. Generally, for a given species and set of conditions the mortality was higher for naiads in the lower (pool) troughs. Most of this difference may be attributed to the difference in water velocity. Other things being equal, the lower flow results in a higher TL_m as was subsequently discovered. The lower, broken-line

Table 9. Summary of Conditions of 14-day Experiments with Unpolluted River Water in Artificial Streams.

Expr. No.	Begun	Type	Bott. Type	Surf. Veloc. ft/sec	Temp. Range °C.	Recorded Range of Diss. Oxygen, mg/l — Above Rocks Min.	Above Rocks Max.	Under Rocks Min.	Under Rocks Max.	Remarks
I-2	6/21/58	riffle	2	0.4	20.2-26.0	4.80	7.20	4.42	6.88	deoxygenated
I-3	6/22/58	do	do	do	20.5-27.0	3.36	5.30	3.10	7.10	do
I-5	do	do	do	do	20.0-26.5	6.16	6.50	5.76	6.72	control
I-B	do	pool	1	nil	20.5-26.0	5.06	6.00	4.88	7.04	deoxygenated
I-C	6/21/58	do	do	do	21.0-24.5	3.70	4.20	3.50	7.40	do
I-E	do	do	do	do	20.5-26.0	6.72	7.10	6.18	7.20	control
II-2	7/14/58	riffle	2	0.4	23.7-28.2	4.24	5.18	4.66	4.82	deoxygenated
II-3	do	do	do	do	24.5-28.0	2.49	4.40	2.92	4.42	do
II-5	do	do	do	do	24.0-29.0	5.00	5.90	5.78	5.80	control
II-B	do	pool	1	nil	24.0-28.0	4.20	5.10	4.20	4.76	deoxygenated
II-C	do	do	do	do	25.5-26.9	2.32	4.50	2.18	3.84	do
II-E	do	do	do	do	24.0-26.9	5.32	6.10	5.86	6.14	control
III-2	8/11/58	riffle	2	0.4	25.0-26.5	3.20	4.06	2.72	3.24	deoxygenated
III-3	do	do	do	do	26.0-27.0	2.22	2.70	-	-	do
III-5	do	do	do	do	24.5-26.5	7.44	7.84	-	-	control
III-B	do	pool	1	nil	24.0-26.0	3.00	3.68	-	-	deoxygenated
III-C	do	do	do	do	24.0-26.5	2.40	2.70	-	-	do
III-E	do	do	do	do	24.0-26.0	6.68	7.88	-	-	control

Table 10. Summary of Results of 14-day Experiments with Unpolluted River Water in Artificial Streams.

Exper. No.	Minimum Recorded D.O.[1]	*A. californica* In	Out	Per cent Survival	*A. pacifica* In	Out	Per cent Survival	Limnephilid[3] In	Out	Per cent Survival	*Pteronarcys* sp. In	Out	Per cent Survival
I-2	4.42	20	16	80.0	-	-	-	30	19	63.3	-	-	-
I-3	3.10	28	16	57.1	-	-	-	30	13	43.3	-	-	-
I-5	5.76	-	-	-	20	12	60.0	30	23	76.7	-	-	-
I-B	4.88	-	-	-	20	8	40.0	30	23	76.6	-	-	-
I-C	3.50	-	-	-	20	10	50.0	30	11	36.7	-	-	-
I-E	6.18	-	-	-	20	8	40.0	30	21	70.0	-	-	-
II-2	4.24	30	17	56.7	10	8	(80.0)[2]	30	28	93.3	20	18	90.0
II-3	2.49	30	13	43.3	10	2	(20.0)[2]	30	21	70.0	20	9	45.0
II-5	5.00	30	16	53.3	10	8	(80.0)[2]	30	27	90.0	20	16	80.0
II-B	4.20	30	18	60.0	10	2	(20.0)[2]	30	26	86.7	7	4	57.1
II-C	2.18	33	14	42.4	10	0	(0.0)[2]	30	17	56.7	7	0	0.0
II-E	5.32	30	18	60.0	10	4	(40.0)[2]	30	29	96.7	7	6	85.7
III-2	2.72	20	16	80.0	10	2	(20.0)[2]	20	14	70.0	10	9	90.0
III-3	2.22	20	8	40.0	10	0	(0.0)[2]	20	2	10.0	10	9	90.0
III-5	7.44	20	17	85.0	10	6	(60.0)[2]	20	20	100.0	10	8	80.0
III-B	3.00	20	12	60.0	10	1	(10.0)[2]	20	13	65.0	10	6	60.0
III-C	2.40	20	4	20.0	10	0	(0.0)[2]	20	5	40.0	10	2	20.0
III-E	6.68	20	17	85.00	10	5	(50.0)[2]	20	20	100.00	10	9	90.0

1 Dissolved oxygen in mg/l.
2 Naiads in screen capsules.
3 Larvae in troughs for 10 days only.

portion of the curve in Figure 18 was drawn on the strength of data on the survival of A. pacifica in capsules shown in Figure 20, and of data on A. californica from tube experiments to be presented later. A comparison of Figures 19 and 20 might lead to the conclusion that naiads of A. pacifica are less tolerant than naiads of A. californica to reduced dissolved oxygen. However, the curve in Figure 19 for A. pacifica is based mostly on data obtained with naiads which were held in screen capsules in the troughs. It is realized that the conditions in the capsules were not identical with those in the troughs, so that the curves in Figures 19 and 20 are not strictly comparable.

The lowest recorded oxygen concentration-survival curves for Acroneuria in unpolluted water, water receiving sulfite waste liquor, and water receiving kraft waste in pool and riffle experiments are compared in Figure 21. It is readily apparent that reduced dissolved oxygen alone is not responsible for the deaths of naiads in the streams receiving sulfite and kraft liquor. Also, it seems, from these curves alone, that in the experiments with kraft liquor, there were some causes of death which were absent or less important in the experiments with sulfite liquor. The same conclusions may be drawn from the results with Pteronarcys naiads and limnephilid larvae

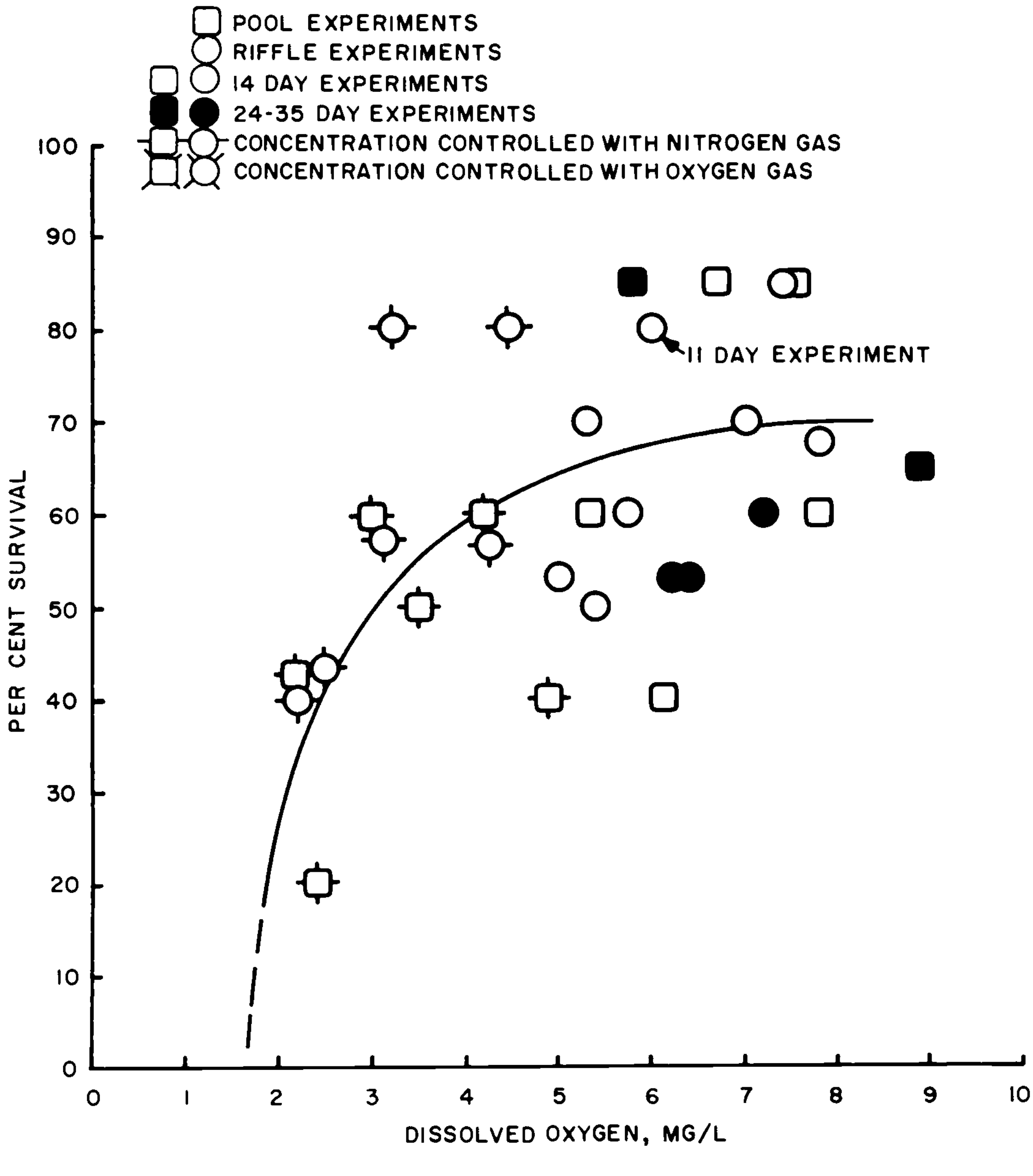

Figure 18. Survival of Acroneuria at lowest recorded concentrations of dissolved oxygen in experiments involving no waste liquor. Data obtained with animals in capsules not included.

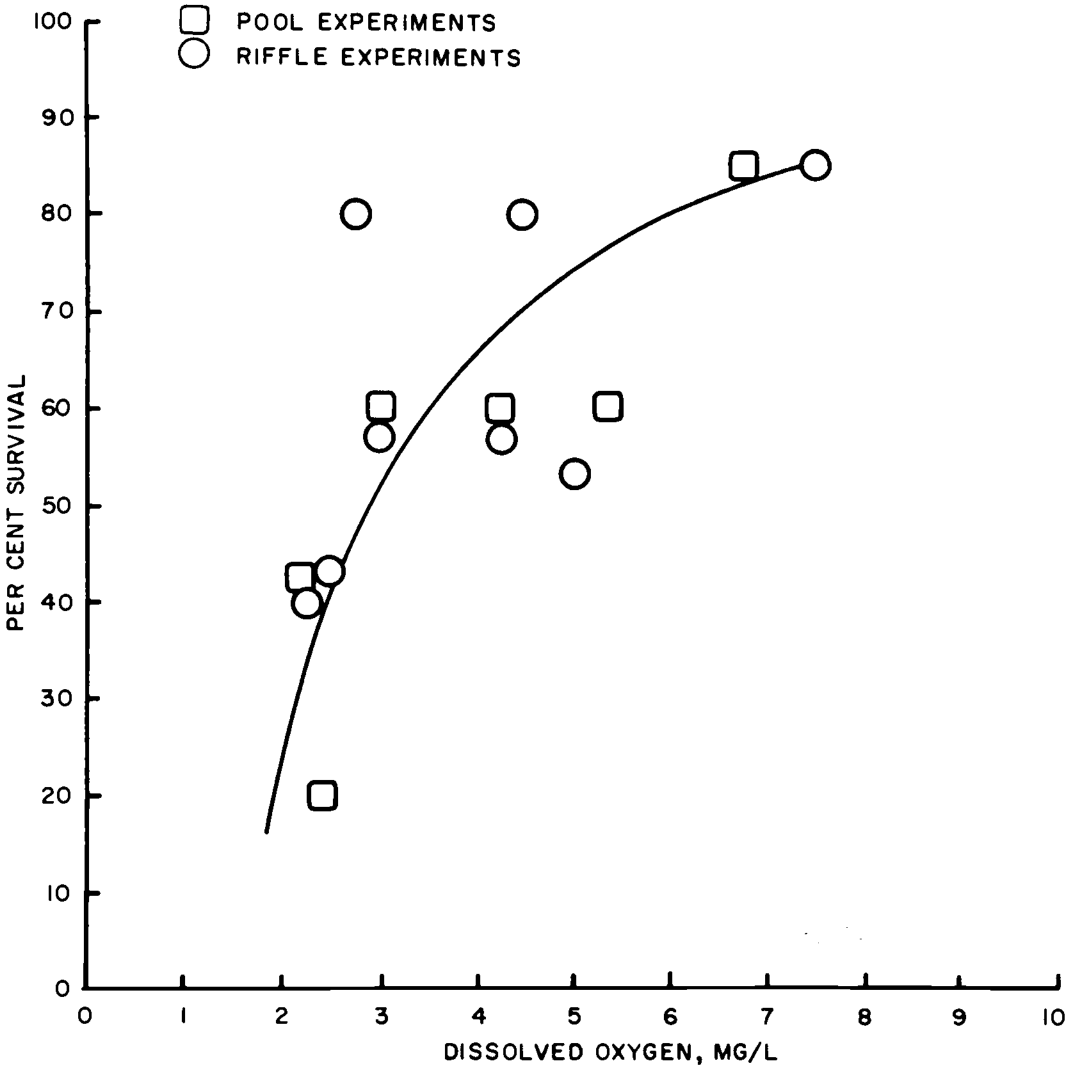

Figure 19. Survival of A. californica at lowest recorded concentrations of oxygen in 14 day experiments with unpolluted river water.

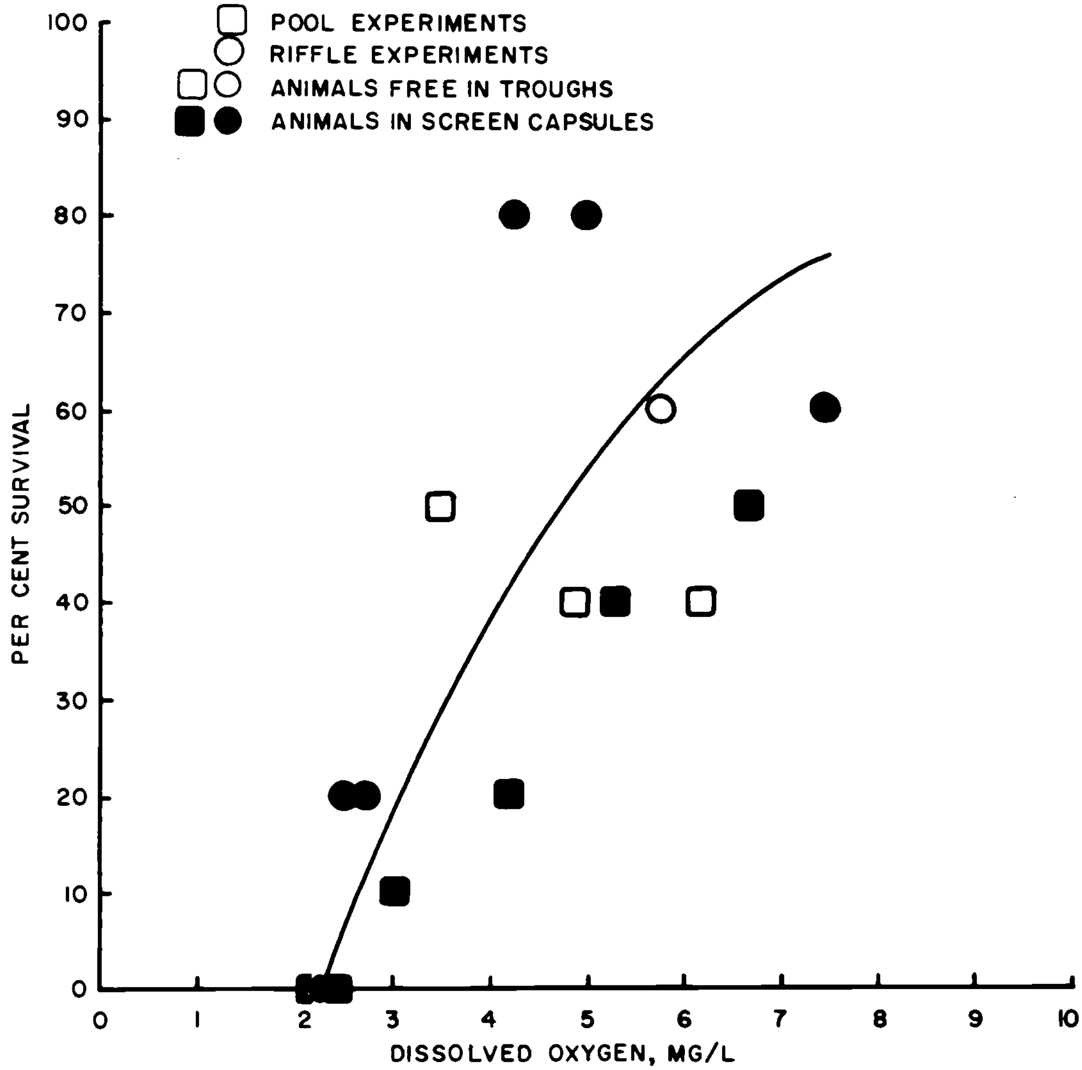

Figure 20. Survival of A. pacifica in relation to lowest recorded concentrations of oxygen in 14-day experiments with unpolluted river water.

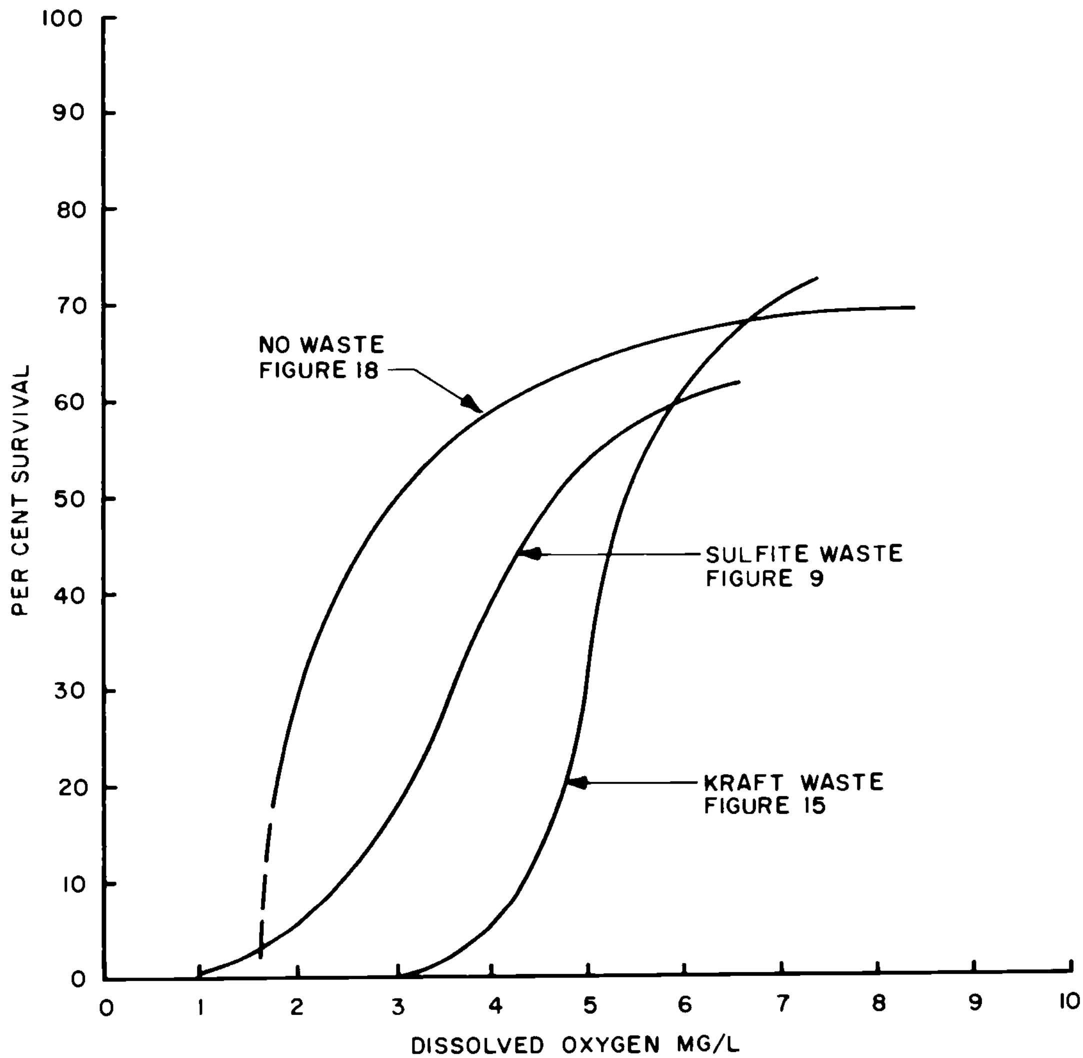

Figure 21. Comparison of concentration-survival curves for Acroneuria for experiments with sulfite waste, kraft waste, and unpolluted water.

plotted in Figures 22 and 23, respectively. However, the data on limnephilids pertain to sulfite waste only.

The curves for sulfite and kraft waste and the unpolluted water curves should be compared with caution, as the results of determinations of dissolved oxygen by the use of the Alsterberg (azide) modification of the Winkler method are subject to more or less interference by these wastes. There was a 0.40 mg/l difference only in the dissolved oxygen values (9.50 and 9.10 mg/l) determined for water of a single water sample (blank) and a duplicate sample of water containing 0.66 per cent prepared kraft waste from sample No. 10 (see Table 2). The Alsterberg modification of the Winkler method was used for these determinations.

Wilbur P. Breese, however, observed (6) considerable apparent interference by a kraft waste. He obtained with the Alsterberg (azide) modification of the Winkler method, dissolved oxygen values which averaged 0.64 to 2.30 mg/l (mean, 1.55 mg/l) lower, for 8 per cent concentrations of kraft waste and oxygen concentrations averaging 5.49 mg/l, than the values obtained with the use of Ohle's (34) "iodine-difference" method. Using the Alsterberg method, I obtained dissolved oxygen values 1.7, 1.3, and 0.6 mg/l lower for river water containing 800, 800 and 400 mg/l of sulfite waste, respectively, than for river water

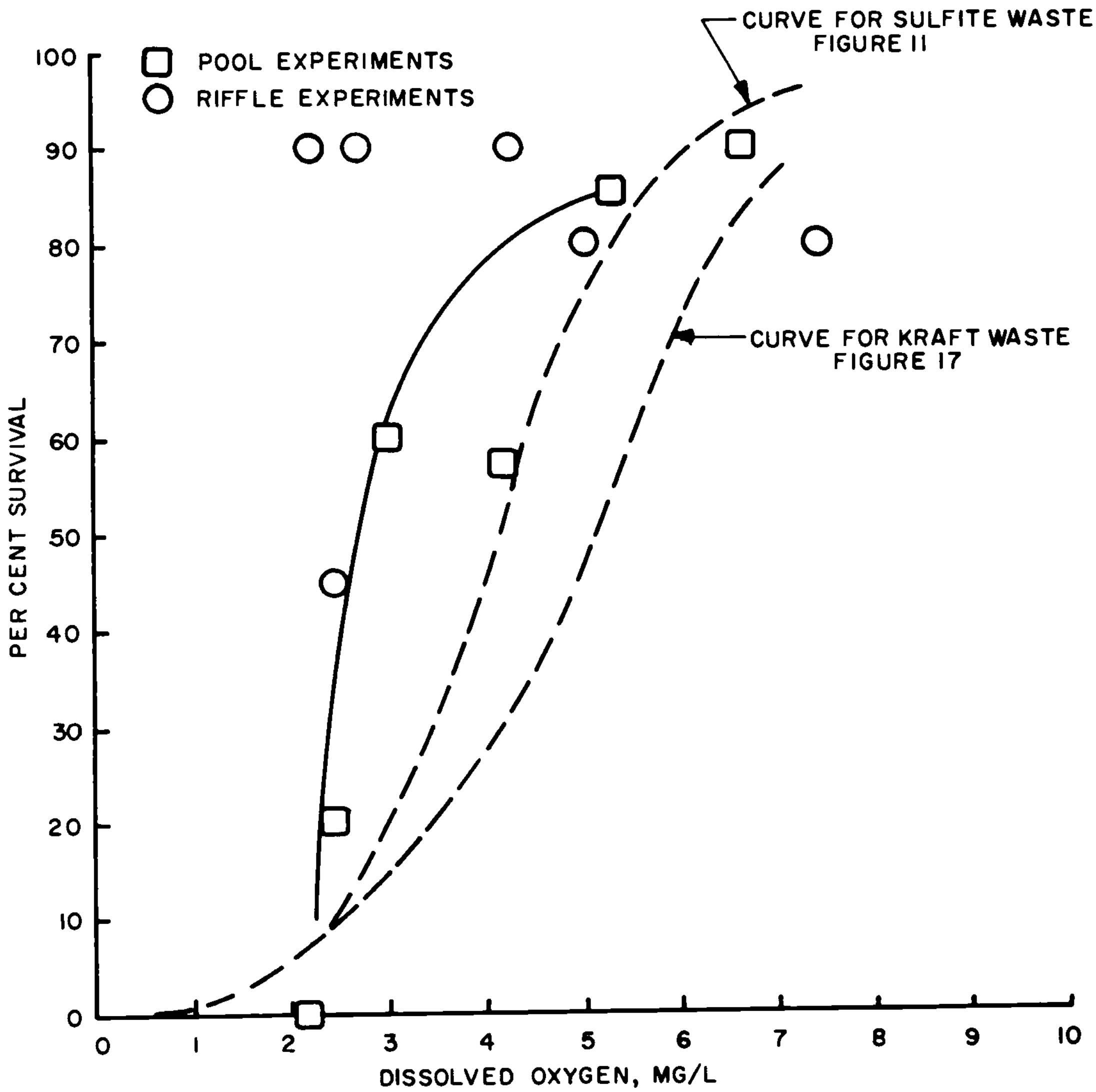

Figure 22. Survival of Pteronarcys at lowest recorded oxygen concentrations in 14-day experiments with unpolluted river water.

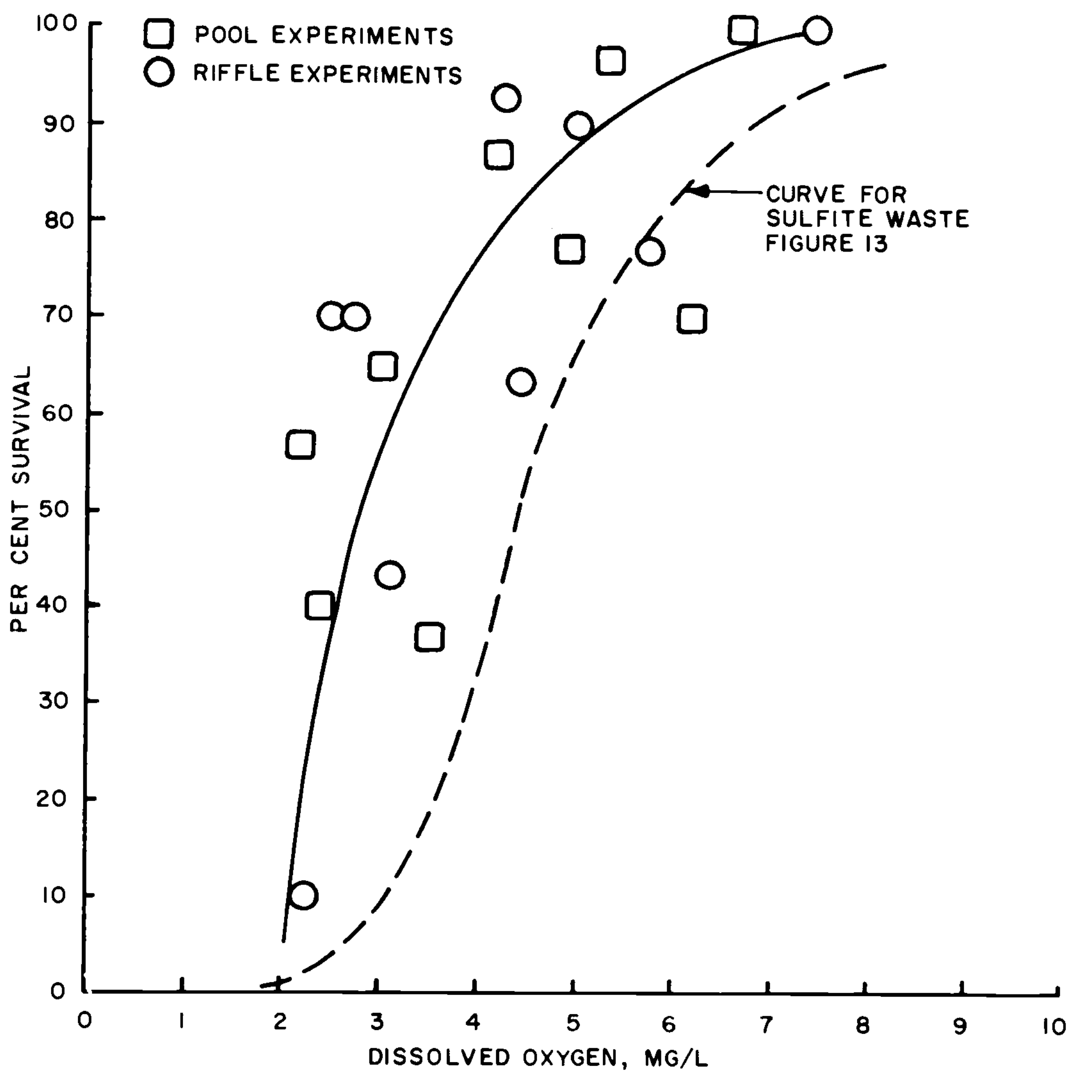

Figure 23. Survival of limnephilid larvae from Alsea River at lowest recorded oxygen concentrations in 10-day experiments with unpolluted river water.

sample blanks having oxygen concentrations of 7.8, 6.3, and 5.5 mg/l, respectively. In the presence of sulfite waste (perhaps comparable to that used in the stream experiments) at a concentration of 500 mg/l in sea water, Breese obtained by Alsterberg method, dissolved oxygen values which averaged 1.42 mg/l lower than the values obtained by the "iodine-difference" method, with the dissolved oxygen ranging from 7.32 to 10.62 mg/l.

Breese's dilutions were prepared with sea water (salinity, 20 parts per thousand) and with kraft waste very different from that used in the stream experiments, so that any estimate of the amount of interference that occurred in the present experiments with either sulfite or kraft waste, based on his results, may be invalid. Furthermore, there is still some question as to the complete reliability of oxygen determinations made by Ohle's iodine difference method in the presence of these wastes.

It is still not clear exactly how the curves in Figures 21, 22 and 23 should be adjusted to reflect the real relationships between dissolved oxygen and mortality. The sulfite curves should be farther to the right, perhaps to the extent of about 1.0 mg/l at the mid-point of the curves. The kraft waste curves perhaps also should be displaced to the right, but only slightly so. If such adjustments were made, the curves would indicate only little or no difference between the effects of sulfite and kraft waste on the animals. The differences between the curves for the non-pollutional experiments and the ones involving either waste would be increased by the adjustment. Possibly, the sulfite waste, although not being nearly as toxic as kraft waste in the presence of abundant dissolved oxygen, somehow has a greater effect on the dissolved oxygen requirements of Acroneuria than does kraft waste, when the concentrations of the two wastes are such that the dissolved oxygen concentrations are equally depressed.

It is, therefore, evident that reduced oxygen alone could not be responsible for the increased mortality observed among naiads of Acroneuria and Pteronarcys, and limnephilid larvae in artificial streams receiving sulfite or kraft waste. The increased mortality which was not due to oxygen lack alone must have been due mainly to toxicity of some waste components, to lowering of the tolerance of the animals to reduced dissolved oxygen by some substances in the wastes, to other interactions, or to combinations of these.

The importance of factors such as the temperature and the velocity of the water, which markedly influence the tolerance of the animals to reduced dissolved oxygen, needs to be known before losses due to insufficient oxygen, even in the absence of wastes, can be fully understood. Where other important causes of death may be involved, full understanding of their actions and interactions obviously requires much more information of this kind.

There was not time to investigate all the causes of loss and since dissolved oxygen proved to be one of major consequence in the artificial stream experiments, it was decided that most supplementary work would be on oxygen tolerance. This work was done using *Acroneuria* naiads as the experimental animals because most of the results just reported dealt with them and because they are widely distributed and are available the year-around. In the section to follow, the results of experiments to determine the tolerance of *Acroneuria* to low dissolved oxygen under different conditions of temperature and water velocity are reported. Also considered are some relationships between naiad behavior and physiology and the concentration of dissolved oxygen, the water temperature, and the water velocity.

APPARATUS, MATERIALS, AND METHODS FOR TUBE AND JAR EXPERIMENTS

The closed recirculation apparatus

Figure 24 shows diagrammatically a single unit of the apparatus which was used for determining the tolerance of Acroneuria naiads to low oxygen concentrations under different conditions of temperature and flow in unpolluted water. Water temperatures were held within about 1°C. of the desired levels by placing the apparatus in a constant temperature water bath or in a constant-temperature room. Generally, the apparatus consisted of a small centrifugal water pump with polyethyline housing and a rubber impeller which pumped the test solution through Tygon tubing from a large carboy (equilibration chamber) through a 5-foot glass pipe with an inside diameter of 2-1/2 inches. Dissolved oxygen concentrations were held within about 0.25 mg/l of the desired levels by continuously bubbling an appropriate mixture of nitrogen and oxygen gas into the carboy by means of air-stones.

The glass pipe served as the testing chamber, and test animals were held in it in a capsule of stainless steel screen (Figure 25). The bodies of the capsules were held away from the sides of the pipe by springs. The flow of water was along and to some extent through

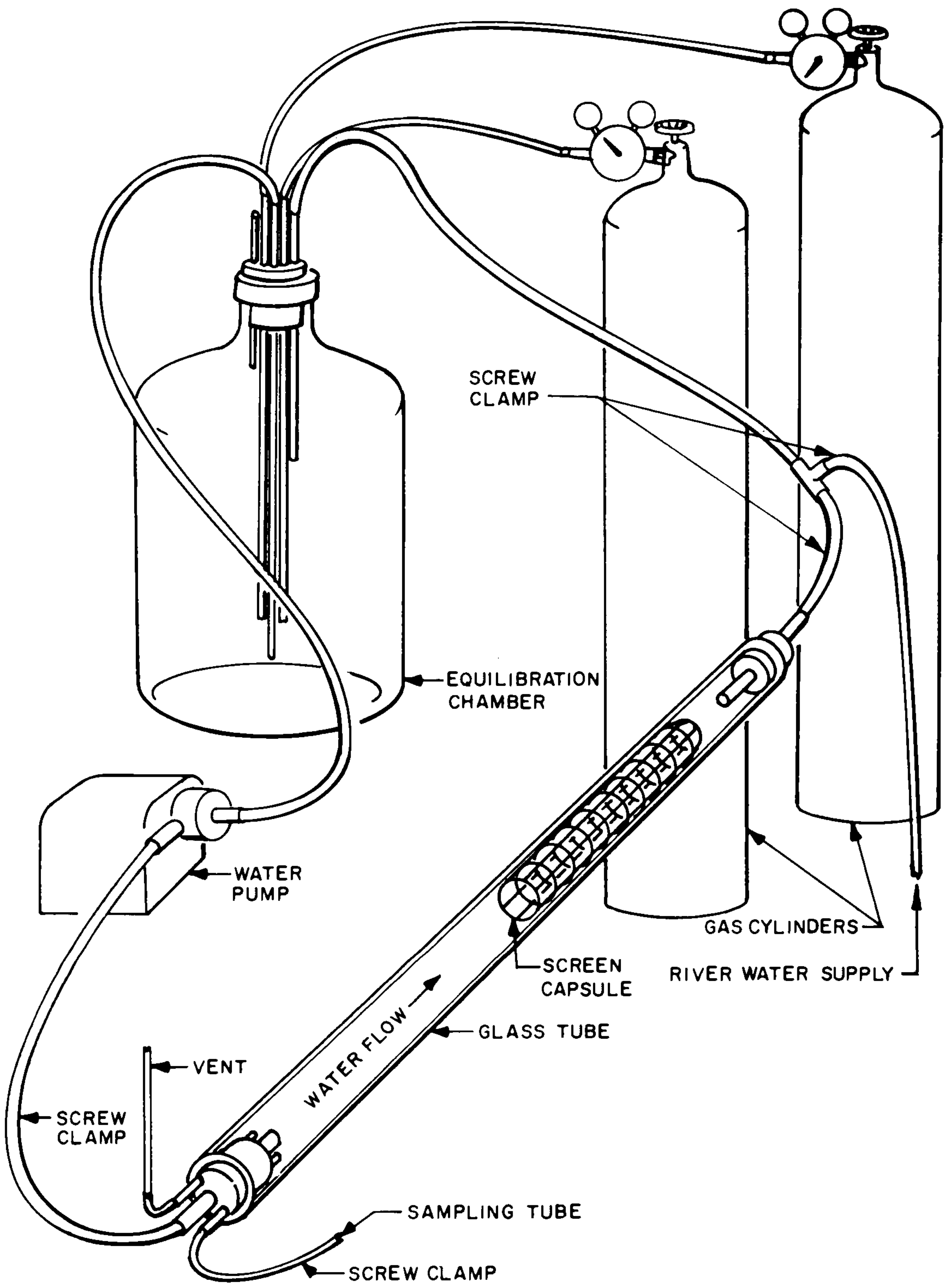

Figure 24. Diagram of one unit of the closed recirculation apparatus (not to scale).

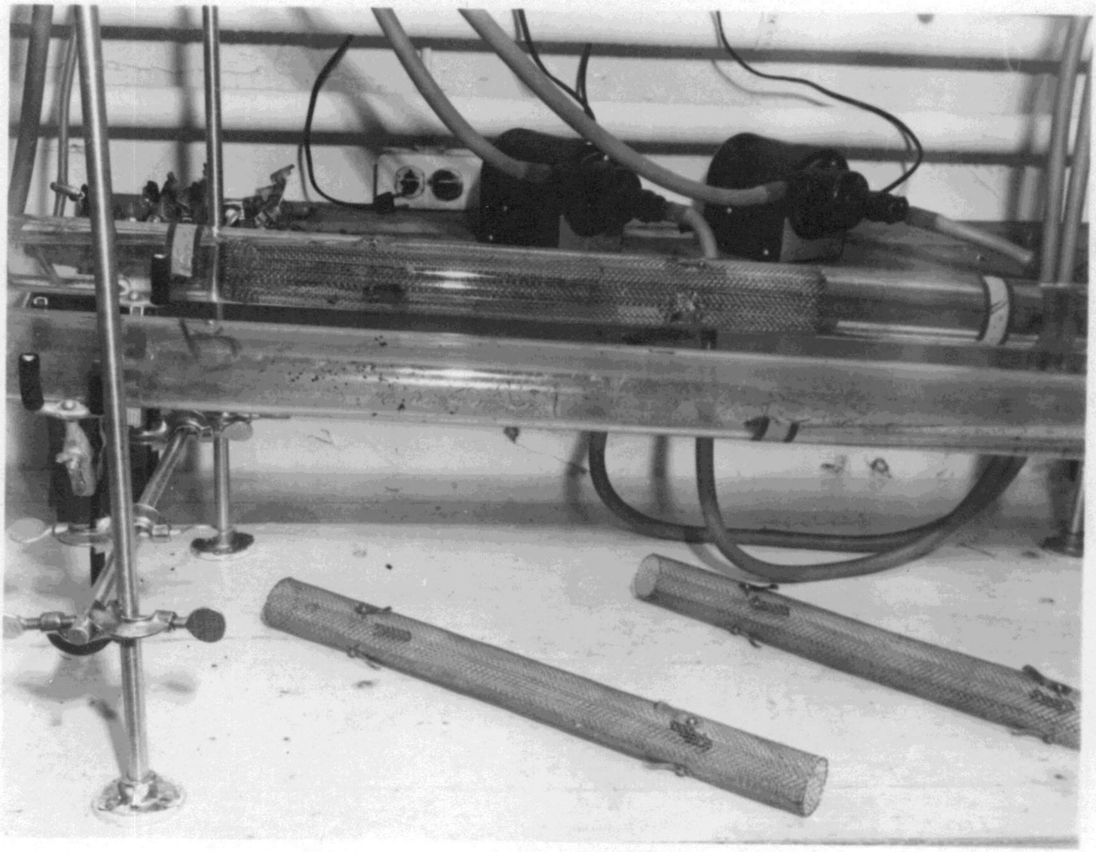

Figure 25. Showing screen capsules and their placement in the glass pipe.

the meshes of the walls of the capsules. The observed movement of particles suspended in the water indicated no zones of stagnation of flow anywhere in the capsules. Screw clamps on the tubing at each end of the pipe provided the means of adjusting the velocity of the water passing through the pipe. It was necessary to divide the discharge from one pump between two pipes in order to maintain, within close limits, very low velocities. Water samples for chemical and physical analysis were drawn from the pipe at the inflow end through a piece of glass tubing with a clamped section of Tygon tubing at its end. As any material change in the water level in the equilibration chamber ultimately altered the oxygen concentration in the circulating water, a withdrawal of a sample of water for analysis was immediately followed by a replenishment with a like volume of stock water of the same temperature.

In preliminary trials with this apparatus constructed with latex tubing, the test animals died or became moribund in a few hours under the most favorable conditions which could be obtained and no test results were valid. Subsequent testing of all parts of the apparatus proved conclusively that the latex tubing was responsible for the difficulty (De Witt, 11, p. 840). Tygon tubing was found to be suitable and all results reported here are based on experiments run in an apparatus constructed only with this tubing.

All experiments were run under conditions of continuous natural or artificial illumination. No information was found on activity or metabolic rhythms in stoneflies; however, Harker (24) reported that established diurnal activity rhythms of mayfly naiads of several species were maintained even under abnormal conditions of light, including reversed diurnal illumination.

Ambuhl (2, p. 223, 224) used for his determinations of low oxygen tolerance in flowing water a glass "circulation pipe" (originally described by Dr. O. Jaag in 1955, according to Ambuhl) in which 90 ml. of water could be kept in motion by a motor-driven propeller. His test animals were not kept in capsules, but they were provided with a "foot-hold" on the inner surface of the lower half of the pipe by sanding with powdered glass. As in the present study, experiments were conducted by Ambuhl under conditions of continuous illumination.

Apparatus for standing water experiments

Where no current was desired, experiments were run in tall 6-liter glass specimen jars held in a constant-temperature water-bath or a constant-temperature room. For oxygen tolerance and some ventilation rate experiments, each jar was supplied with lines of Tygon tubing from cylinders of nitrogen and oxygen gas for use in adjusting the concentration of dissolved oxygen. The test animals were held in the jars in capsules of aluminum or stainless steel screen.

For oxygen consumption experiments, the test water was brought to the desired oxygen concentration by bubbling nitrogen and oxygen gas into water in a large carboy. The test solution was then siphoned into the test containers. Exchange of oxygen between the air and the solution was largely prevented by a 1-inch layer of mineral oil, which was poured on the surface of the test solution after insertion of the test animals and siphons for water sampling and water addition.

The test animals and their acclimatization

The test animals, *Acroneuria pacifica* Banks and *Acroneuria californica* (Banks), are large stoneflies, carnivorous as naiads, which are very common in large and small streams from British Columbia to California. *A. pacifica* is also found from Montana to Colorado (Jewett, 30, p. 89). The naiads of *A. pacifica* possess comspicuous anal gills which are entirely absent in naiads of *A. californica*. Also, the naiads of *A. pacifica* are noticeably darker than those of the latter species. The naiads of one other species, Acroneuria theodora Needham and Claassen, found in the same general areas, might be confused with those of A. californica, except that their head markings and color patterns are distinctive.

Both species of test animals possess tufts of hollow, finger-like thoracic tracheal gills as external parts of their closed respiratory systems. These gills are thin-walled evaginations of the integument and increase the surface available for the exchange of respiratory gases. The gills connect with a network of fine branched tracheae lying beneath the integument. The tracheae end in branched tracheoles which are finer tubes less than 1 micron in diameter and extending to most parts of the body. Respiratory gases pass by diffusion to and from the tissues via the tracheoles, the tracheae, and the gills. The functioning of the closed respiratory system depends on transfer of gases by passive diffusion between the system and the ambient medium. Perhaps only 10 or 12 minutes are required for the gases in the tracheal system of some aquatic insects to come into equilibrium with the gases in the environment (Roeder, 39, p.49). Respiration may occur with or without the aid of mechanical ventilation, and many immature aquatic insects ventilate only sporadically as the need arises, e.g., during periods of increased metabolism.

All or part of the external respiration of aquatic nymphs may be through the integument, but the relative importance of gills and integument in the respiration of the present test animals is not known. The gills of some immature insects apparently are not necessary for respiration. This has been found to be true of a caddisfly (Morgan and O'Neil, 33) and of a mayfly (Wingfield, 55).

While their exact age was not known, all naiads of Acroneuria used in these experiments were believed to be either in their last few months of life (as indicated by subsequent emergence of representatives) or in the year preceding their (spring) emergence. Most naiads were in the wet weight range of 60 to 220 mg.

Generally, all test animals were held under laboratory conditions for about seven days before they were used in an experiment. They were gradually adjusted during this period to the temperature to be maintained in the anticipated experiment. This period was somewhat arbitrarily selected in the absence of information on the length of time required for acclimatization of Acroneuria to temperatures or oxygen concentrations either higher or lower than those prevailing in the environment at the time of collection of the test animals. While it is well established that the thermal history of aquatic insects has an important bearing on the oxygen requirements of these animals, there apparently should be no great concern about the sufficiency of a 7-day period for acclimatization to either a higher or lower temperature insofar as oxygen consumption is concerned. Pattee (35) concluded that only six or seven hours was adequate. Other authorities cited by Pattee recommended periods up to 24 hours. Whitney (53) in his work with naiads of a mayfly concluded that acclimatization to a higher temperature required longer than 40 hours, without providing a clue as to how much longer. The test animals used in the present experiment were held after collection in water maintained at an oxygen concentration near air-saturation by aeration. The test animals fed to some extent on each other and on other organisms living on the vegetation kept in the stock animal containers.

The early tube experiments were carried out at Oregon State University, using Acroneuria of two species from streams in the Corvallis, Oregon, area. These animals are designated either "A. pacifica (Corvallis)," or "A. californica (Corvallis)." It became necessary to shift the investigational work to Arcata, California, and most of the tube experiments were carried out there, using A. californica collected from that vicinity. These particular insects are designated "A. californica (Arcata)."

General procedure for oxygen tolerance experiments

In these experiments the recirculation apparatus was used for all flowing-water tests. In preparation for an experiment, the water bath or the constant temperature room was brought to the desired temperature, the recirculation system filled with well-aerated river water, and the water pump started. When the test water attained the correct temperature, the acclimatized animals were placed in the capsules in the lower end of the testing chamber. The velocity of the flow at the center of the pipe was then adjusted to the desired value.

Velocity measurements were made by timing with a stopwatch suspended particles as they moved near the center of the flow between marks on the pipe two feet apart. As the bodies of the capsules were held away from the sides of the pipe by springs, the naiads in the capsules were positioned fairly well away from the sides of the pipe in the vicinity of which the flow was markedly impeded. Numerous timing indicated that there was no great difference between velocities measured near the center of the pipe and in the vicinity of the naiads clinging to the capsule walls inside the capsule. Of all the variables encountered in this study, the maximum water velocity in the tubes was easiest to measure precisely, there having been only slight variation in replicate measurements.

To begin an experiment, the bubbling of air, which maintained the oxygen concentration at about air-saturation, was discontinued. Nitrogen and oxygen gas were bubbled into the water in the equilibration chamber at appropriate rates, to begin the lowering of the oxygen concentration to the desired test level. The period of oxygen reduction was more or less arbitrarily set at five hours to prevent shock due to too rapid a change in conditions. The final oxygen concentration was maintained for 24 hours. Thus, a total of 29 hours was usually required for the preliminary and final phases of a test. A few longer tests were conducted for special purposes. Ambuhl (2, p. 224) subjected his test animals to a 2-hour or longer adjustment period at a high oxygen concentration in this circulation pipes before a test was begun. His tests ordinarily lasted 6 to 8 hours.

Experiments on oxygen tolerance under standing water conditions were carried out in about the same manner, but 6-liter specimen jars instead of glass pipes served as the testing chambers, and the wire capsules containing the test animals were suspended vertically in the jars.

In most cases 10 to 20 animals were used per test. The number of animals which survived each test was recorded along with information on the behavior of the surviving naiads and that of the others up to the time of death. The criterion of death was immobility and absence of any response whatever on probing over a period of a minute or more. Immobility apparently was used as the criterion in Ambuhl's (2, p. 225) work also.

It is realized that "death" and "immobility" are not truly synonymous terms. The use of immobility as the criterion in experiments at the temperature of 2.2°C. resulted in (apparent) mortalities that were too high. An average of 50 per cent of the naiads initially considered dead recovered after being exposed to the air or replaced in air-saturated water for about an hour or more. Brett (7, p. 275) reported similar difficulties in experiments on low temperature tolerance in fish and stated that a lack of suitable criteria has restricted work at this temperature level. At temperatures of 14°C. and higher, the recovery of immobilized naiads was seldom observed.

Twenty-four hour median tolerance limits (TL_m's) were determined graphically from "dose-survival" curves by the method of Litchfield and Wilcoxon (31, p. 99-113). Their methods of testing for "goodness of fit" of the curves to the data and for determining confidence limits at the 95 per cent level of significance for TL_m's were also employed. Eisenberg (16, p. 120-121) has shown that this method will not underestimate the size of confidence intervals obtained by the standard method of Bliss (5, p. 192-216). The determination of TL_m's by the method of Litchfield and Wilcoxon involves the use of log-probit paper. By obtaining "corrected" (table) values for some 100 and zero per cent survival observations, these observations may be taken into account in plotting the "dose-survival" curves to better define the slopes of the curves. A maximum of only two consecutive 100 per cent and two zero per cent survival observations may be used in this way, and only one of each of such observations made were used in plotting the experimental data. Each corrected per cent survival value used in plotting the curves is indicated on the graphs relating dose to survival (Figures 27 to 36) by a square rather than a circle.

In the tube experiments there was relatively little cannibalism and it could usually be observed when it did occur. Results of experiments in which cannibalism was known to occur were discarded. Except for cannibalism, there were no known causes of mortality other than reduced dissolved oxygen; therefore, reliable estimates of median tolerance limits are believed to have been obtained. The survival of control animals was 90 or 100 per cent except for one experiment at 24°C. in still water, in which the survival was 80 per cent. Almost all losses among control animals were known or believed to have been due to cannibalism.

General procedure for oxygen consumption experiments

The water for use in the experiments to determine rates of oxygen consumption was brought to the desired temperature and the screen capsules containing the test animals were placed in it. When tests were to be performed at oxygen concentrations below air saturation, the concentration was gradually lowered over a period of several hours to the desired level by means of nitrogen gas, and 2.5 or 3.3 liters of the water was siphoned into each of two containers. The capsule containing the acclimatized test animals was then placed in one of these containers. The other container was used as a blank for determination of the "biochemical oxygen demand" (B.O.D.) of the water. Then sampling tubes were inserted into both containers, and the surface of the water was covered with a 1-inch layer of mineral oil. After about 15 minutes (when it appeared that the animals had quieted) a sample of water was drawn from each container and determinations of the initial oxygen concentrations were made. Frequently during the subsequent test period the capsules were gently lifted and lowered with a nylon thread to mix the water thoroughly without noticeably disturbing the test animals.

After three hours the dissolved oxygen concentrations in both the test container and in the blank were again determined. The B.O.D. in the test container was assumed to be identical to that in the control container without animals where it was actually measured. After allowing for the B.O.D., the approximate consumption of oxygen by the animals, in mg. of oxygen per gram of dry animal weight per hour, was determined. The mean of the initial and final dissolved oxygen values was considered to be the concentration at which the oxygen consumption rate was determined. The dry weight of the animals was determined after drying them for 48 hours at 105°C.

No allowance was made for the possible reduced consumption of oxygen by naiads which became immobilized because of deficiency of oxygen before the completion of a test. Figure 26 shows that at 20°C. the naiads continue for some time after immobilization to consume measurable quantities of oxygen. In the case illustrated, the reduction in the dissolved oxygen concentration in container No. 2 was 0.90 mg/l for the 4-hour period just after all naiads became immobilized. In container No. 1, the reduction was only 1.5 mg/l for the 4-hour period during which all or nearly all of the naiads were still mobile. In 3-hour experiments, especially where

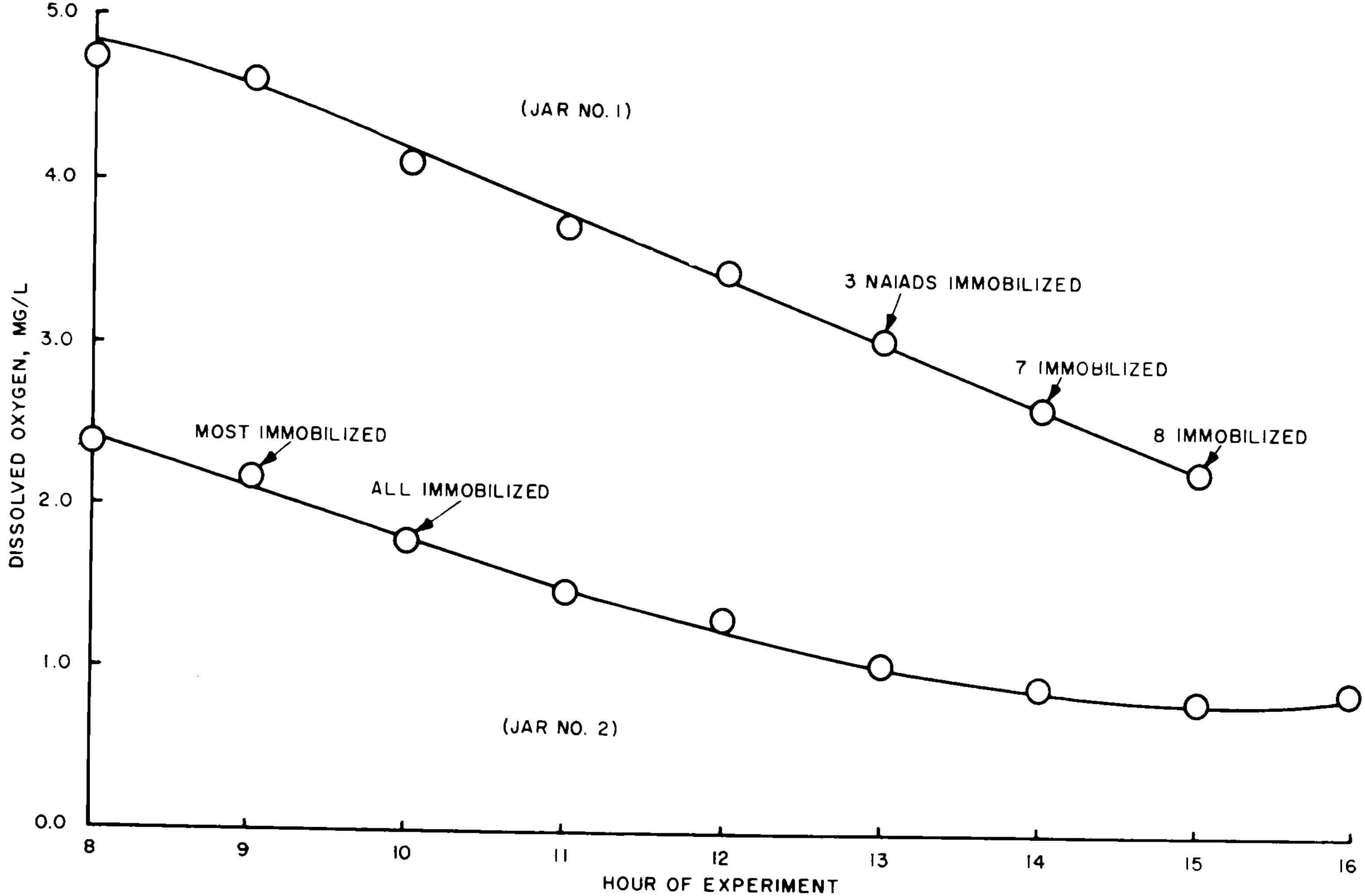

Figure 26. Showing reduction of dissolved oxygen in two jars, each containing 21 naiads of A. californica (Arcata), at 20°C.

immobilization, if it occurs at all, occurs only near the end of the 3-hour period, there is little need to allow for reduced consumption of oxygen by immobilized animals in arriving at reasonably reliable estimates of the consumption per gram of mobile animals.

In all experiments the Alsterberg (azide) modification of the Winkler method for determination of dissolved oxygen was employed. Observations on animal behavior, particularly those movements related to respiration, were made throughout each experiment.

Methods and rationale for counting "push-up" movements

Naiads of *A. californica* and *A. pacifica* engage in rhythmic vertical body movements which are influenced by the concentration of dissolved oxygen and certain factors which determine oxygen requirements and availability, such as water temperature and velocity. There is no doubt that these movements are for the most part ventilatory movements, but even under conditions of oxygen saturation some "push-ups," possibly nervous reactions, occur when the naiads are disturbed. According to Ambuhl (2 p. 243), Barak and Foustka (1907) were the first to establish that ventilatory movements among arthropods are influenced by the oxygen content of the water.

Schoenemund (40) observed these "very peculiar rhythmical motions" of several species of gill-bearing and gill-less stonefly naiads. He noticed also that in rapidly flowing water at a low temperature these movements occurred much less frequently, and he concluded that the movements must be related to respiration.

Pteronarcys naiads engage in horizontal swaying motions which are also apparently influenced by the concentration of dissolved oxygen. While no counts of these movements were recorded, general observations on them at various oxygen concentrations were made.

Push-ups among Acroneuria occur in "bursts" (series) usually lasting 4 to 30 seconds at high and moderately low concentrations of oxygen. At very low concentrations push-ups may be more or less continuous. The wave-like ventilatory movements of some caddisfly larvae also become continuous at very low oxygen concentrations (Van Dam, 48; Fox and Sidney, 17). The intervals between bursts of "push-ups" may be from several minutes to a few seconds, again depending on the level of dissolved oxygen and the physiological needs of the naiads. The amplitude of the push-ups seems to be greater when the bursts are of shorter duration and less frequent, but no measurements have been made.

For the determination of rate, push-ups were counted over short periods during which there was no change in the rhythm of these movements. Only the rates of continuous and regular movement in more or less evenly spaced series are believed to adequately reflect the existing conditions of dissolved oxygen.

The total numbers of push-ups performed either continuously or discontinuously by individual naiads during 5-minute periods sometimes were determined as a measure of over-all ventilatory activity of the animals. These numbers will be referred to as "5-minute total counts." The 5-minute counting period was arbitrarily selected as being long enough for obtaining meaningful counts, but short enough to make possible a suitable number of observations. In two experiments a more or less corresponding measure of the ventilatory activity of the naiads was obtained by multiplying the determined mean push-up rates (push-ups per minute) by the estimated fraction of all test animals that were performing push-ups at any one time. This measure will be referred to as the "weighted push-up rate."

General procedure for determining the effect of velocity on push-up rate

The effect of water velocity on the rate of push-up movements at a constant oxygen concentration was studied using the recirculation apparatus and test animals acclimatized to the test temperatures in air-saturated water. During the special experiments performed for this purpose, the velocity of the water, initially zero, was increased step-wise in small increments at hourly intervals until the maximum velocity to be tested was attained. The push-up rates of those naiads performing push-ups and the percentage of test animals performing these movements regularly during the last 20 minutes of each hourly period were recorded.

RESULTS OF TUBE AND JAR EXPERIMENTS AND RELATED OBSERVATIONS ON THE ARTIFICIAL STREAMS

The 24-hour median tolerance limits of reduced dissolved oxygen at different water velocities and at one temperature level (20°C.) were determined for naiads of A. pacifica (Corvallis) and A. californica (Corvallis). Table 11 and Figures 27 and 28 show the results of these experiments, which were carried out in Corvallis. It was expected that additional results to be obtained in Arcata after relocation of the work would provide, together with these results, the background for a more complete analysis of the results of the work in Corvallis with the artificial streams. However, it soon became evident that there were considerable physiological differences between A. californica naiads from the two areas. Thus, the results of tube tests with A. californica (Arcata) are not the same as those with A. californica (Corvallis), nor can the former results be directly related to the results obtained with A. californica (Corvallis) in the artificial stream experiments.

Table 12 and Figures 29-36 show the results of 70 twenty-four hour experiments to determine the survival of A. californica (Arcata) naiads under different conditions of temperature, water velocity, and oxygen concentration.

Chi-square tests of goodness of fit of the curves to the plotted points indicated that each curve was a good fit, at the 0.05 level of probability, to the points on which each was based. Median tolerance limits derived from the curves in Figures 29 to 36 are shown in relation to test temperature and water velocity in Table 13 and Figure 37.

The highest and lowest temperatures that were tested, 24 and 2.2°C. respectively, are believed to be near the extremes to which naiads of A. californica are exposed ordinarily in the streams from which the test animals were collected. Tests were performed also at 14 and 20°C. The temperature of 24°C. may be near the maximum tolerable by the average A. californica (Arcata) naiad. When the curves in Figure 37 are extrapolated to the right, they converge at a point near 25°C., indicating that the influences of both oxygen concentration and water velocity are overridden by high temperature as the primary environmental factor limiting survival above this temperature level. Whitney (53) found that the median tolerance limit for several species of mayfly naiads from rapidly flowing streams in England ranged from about 21 to 27°C. Whitney also found that increased dissolved oxygen was of little value in increasing survival at high temperatures, and therefore, the death of mayfly naiads at high temperatures was due not to asphyxiation resulting from increased

Table 11. Summary of Conditions and Results of One-Day Tube Experiments with *Acroneuria californica* and *Acroneuria pacifica* Naiads from the Corvallis, Oregon Region.[1]

Date	Species	No. in Test	No. Surv.	Per Cent Survived	Temp. °C	Oxygen mg/l mean	Oxygen mg/l range	Velocity f.p.s.
3/ 2/57	*californica*	10	7	70	19-20	1.70	0.0	0.17
3/ 3/57	do	10	2	20	do	1.40	0.0	do
3/ 5/57	do	10	2	20	do	1.40	0.0	do
4/ 6/57	do	10	0	0	do	1.02	0.4	do
do	do	10	0	0	do	1.02	0.4	do
4/ 9/57	do	10	0	0	do	1.37	0.4	0.25
4/11/57	do	10	9	90	do	2.00	0.3	do
do	do	10	2	20	do	1.48	0.1	do
4/13/57	do	10	8	80	do	2.60	0.4	do
4/14/57	do	10	1	10	do	1.93	0.1	0.06
4/16/57	do	10	0	0	do	2.14	1.0	do
4/17/57	do	10	4	40	do	2.30	0.0	do
4/19/57	do	10	5	50	do	2.27	0.2	do
4/28/57	do	10	10	100	do	3.38	0.1	do
4/ 6/57	*pacifica*	10	5	50	do	1.57	0.1	0.17*
4/ 8/57	do	10	0	0	do	1.19	0.0	0.25*
4/ 9/57	do	10	0	0	do	1.37	0.4	do
4/19/57	do	10	0	0	do	1.78	0.2	0.06*
4/20/57	do	10	10	100	do	3.63	0.3	do
4/27/57	do	10	4	40	do	3.00	0.0	0.05*
7/12/58	do	10	8	80	do	3.50	0.7	0.03
8/ 3/58	do	8	7	88	do	3.88	1.1	do
8/ 6/58	do	8	2	25	do	2.69	1.8	do
8/15/58	do	8	6	75	do	3.19	0.2	do
do	do	8	1	13	do	2.17	0.7	do
8/17/58	do	7	6	86	do	2.88	0.2	do

1 Data are for the first 24 hours of "effective" test time, but some of the tests were continued for special purposes beyond the 24-hour period.

* Insufficient data for determination of TL_m.

Table 12. Summary of Conditions and Results of One-Day Tube Experiments with *Acroneuria californica* from the Arcata, California Region.[1]

Date	Number In Test	No. Survived	Per Cent Survived	Temp.	Oxygen, Mean	mg/l Range	Velocity f.p.s.
10/30/58	10	10	100	2.2°C.	3.62	0.3	0.00
do	10	8	80	do	3.43	0.3	do
do	10	6	60	do	3.06	0.3	do
do	10	6	60	do	2.98	0.3	do
do	10	7	70	do	2.73	0.5	do
do	10	5	50	do	2.71	0.1	do
do	10	2	20	do	2.64	0.1	do
do	10	3	30	do	2.45	0.2	do
11/11/58	10	1	10	do	2.41	0.3	do
11/13/58	10	6	60	do	2.50	0.1	do
8/23/59	10	6	60	do	0.96	0.0	0.03
8/26/59	10	4	40	do	0.79	0.5	do
9/2/59	10	0	0	do	0.78	0.0	do
10/2/59	10	8	80	do	1.13	0.2	do
10/16/59	10	10	100	do	1.06	0.2	do
8/26/69	10	10	100	do	0.79	0.5	0.13
8/30/59	10	0	0	do	0.32	0.2	do
9/2/59	10	8	80	do	0.78	0.0	do
9/21/59	10	6	60	do	0.61	0.1	do
1/8/59	10	7	70	14.0°C.	2.95	0.5	0.00
12/16/58	10	4	40	do	2.58	0.4	do
12/11/58	10	0	0	do	1.88	0.2	do
12/9/58	10	8	80	do	2.96	0.0	do
12/4/58	10	5	50	do	2.25	0.4	do

1 Data are for the first 24 hours of "effective" test time, but some of the tests were continued for special purposes beyond the 24-hour period.

Table 12. (con't.)

Date	Number In Test	No. Survived	Per Cent Survived	Temp.	Oxygen, Mean	mg/l Range	Velocity f.p.s.
12/3/58	10	10	100	14.0°C.	2.99	0.9	0.00
do	10	1	10	do	2.13	0.1	do
1/31/59	10	3	30	do	2.16	0.2	do
11/16/59	10	9	90	do	1.67	0.2	0.03
11/20/59	10	10	100	do	1.38	0.1	do
11/26/59	10	7	70	do	1.34	0.2	do
11/27/59	10	0	0	do	0.95	0.1	do
1/15/60	10	5	50	do	1.26	0.1	do
11/20/59	10	10	100	do	1.38	0.1	0.13
11/26/59	10	9	90	do	1.34	0.2	do
11/27/59	10	9	90	do	0.95	0.1	do
1/15/60	10	8	80	do	1.26	0.1	do
1/17/60	10	5	50	do	0.87	0.1	do
1/7/59	10	1	10	20.0°C.	2.05	0.0	0.00
7/27/59	10	0	0	do	1.73	0.0	do
7/29/59	10	3	30	do	2.38	0.2	do
do	10	4	40	do	2.38	0.2	do
8/2/59	10	10	100	do	4.32	0.0	do
8/12/59	10	8	80	do	3.45	0.5	do
12/5/57	15	15	100	do	2.39	0.6	0.13
12/10/57	13	10	76	do	1.23	0.3	do
12/10/57	15	7	47	do	1.23	0.3	do
12/17/57	15	0	0	do	0.60	0.0	do
2/17/58	10	7	70	do	1.96	0.2	do
2/25/58	10	8	80	do	1.94	0.4	do
3/10/58	10	7	70	do	1.24	0.1	do
3/19/58	10	7	70	do	1.69	0.4	do
4/7/58	10	9	90	do	3.32	0.2	do

Table 12. (con't.)

Date	Number In Test	No. Survived	Per Cent Survived	Temp.	Oxygen Mean	mg/l Range	Velocity f.p.s.
2/19/58	10	10	100	do	1.96	0.4	0.25*
3/12/58	10	7	70	do	1.22	0.0	do
4/2/59	5	5	100	24.0°C.	5.48	1.0	0.00
4/5/59	10	0	0	do	2.54	0.0	do
4/26/59	10	5	50	do	3.55	0.2	do
do	10	2	20	do	3.50	0.4	do
4/26/59	10	8	80	do	3.74	0.3	do
5/18/59	10	8	80	do	4.31	0.5	do
do	10	7	70	do	4.32	0.5	do
6/11/59	10	7	70	do	2.75	0.8	0.03
7/5/59	10	0	0	do	2.04	0.4	do
7/8/59	10	1	10	do	2.20	0.1	do
7/13/59	10	9	90	do	3.50	0.0	do
6/11/59	10	9	90	do	2.75	0.8	0.13
7/5/59	10	2	20	do	2.04	0.4	do
7/8/59	10	6	60	do	2.20	0.1	do
7/13/59	10	9	900	do	3.50	0.0	do
7/23/59	10	0	0	do	1.16	0.0	do

* Insufficient data for determination of TL_m.

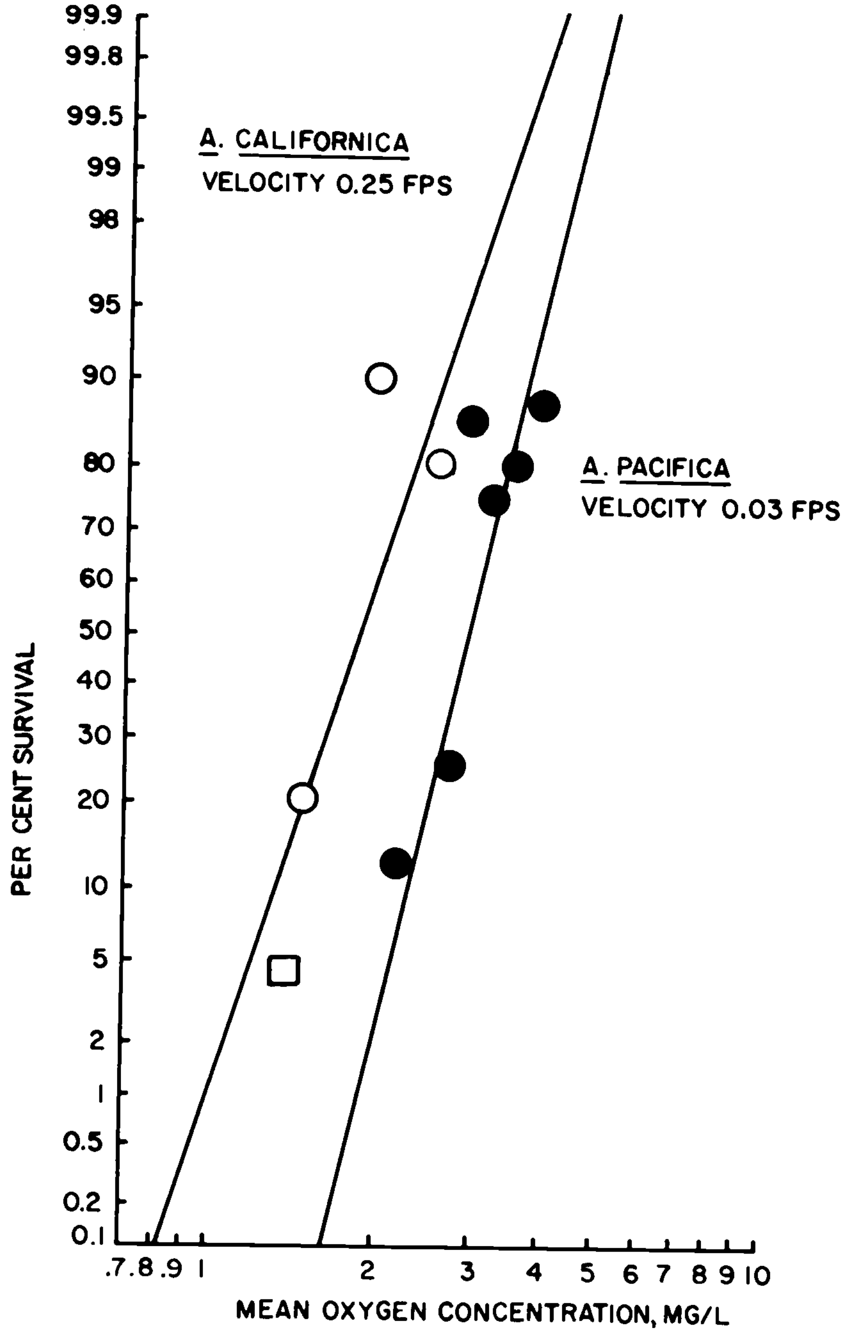

Figure 27. Survival of Acroneuria from the Corvallis, Oregon area, in relation to dissolved oxygen, at 20°C. and at water velocities of 0.03 and 0.25 fps.

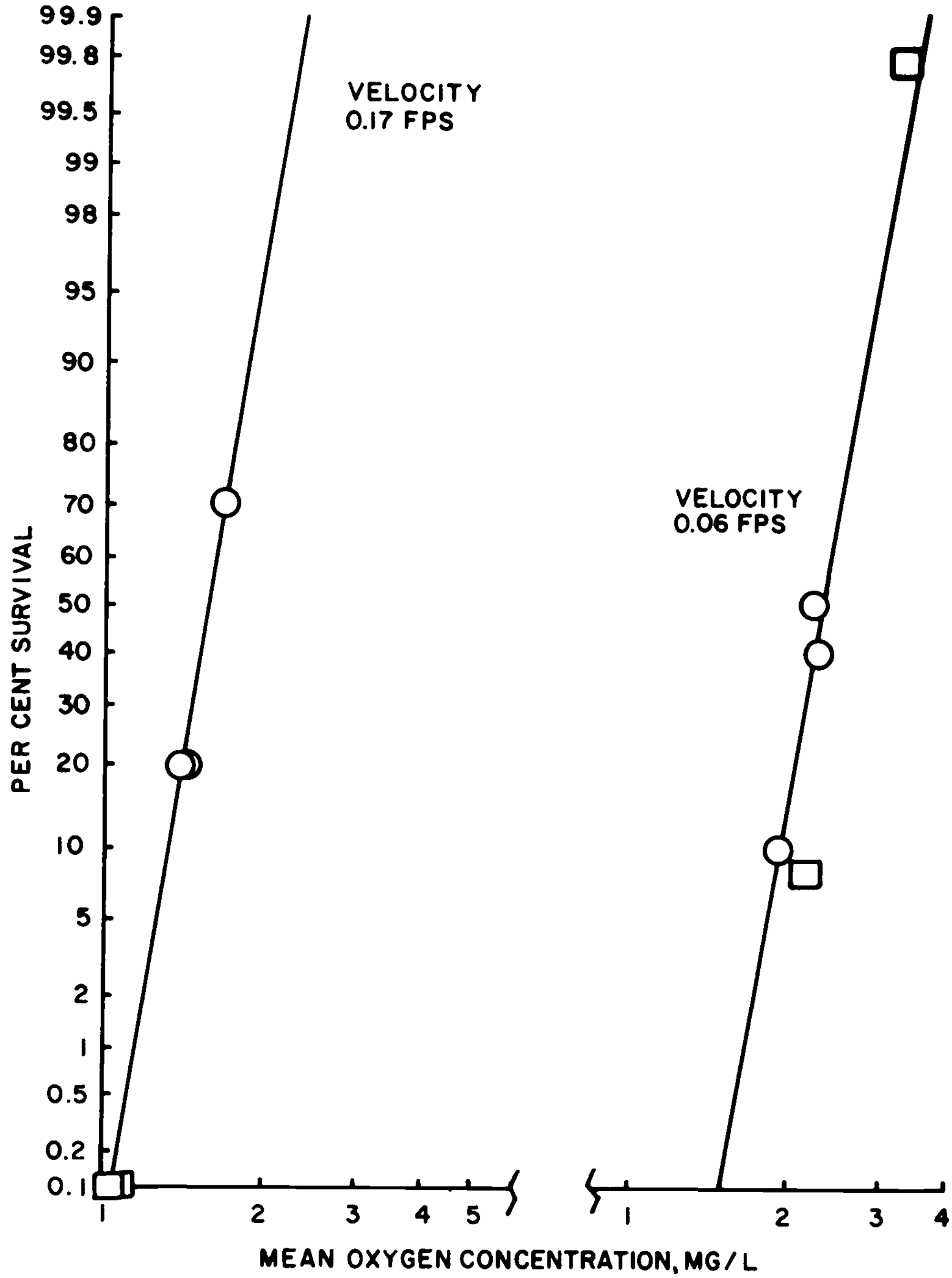

Figure 28. Survival of A. californica (Corvallis) in relation to dissolved oxygen, at 20°C. and at water velocities of 0.06 and 0.17 fps.

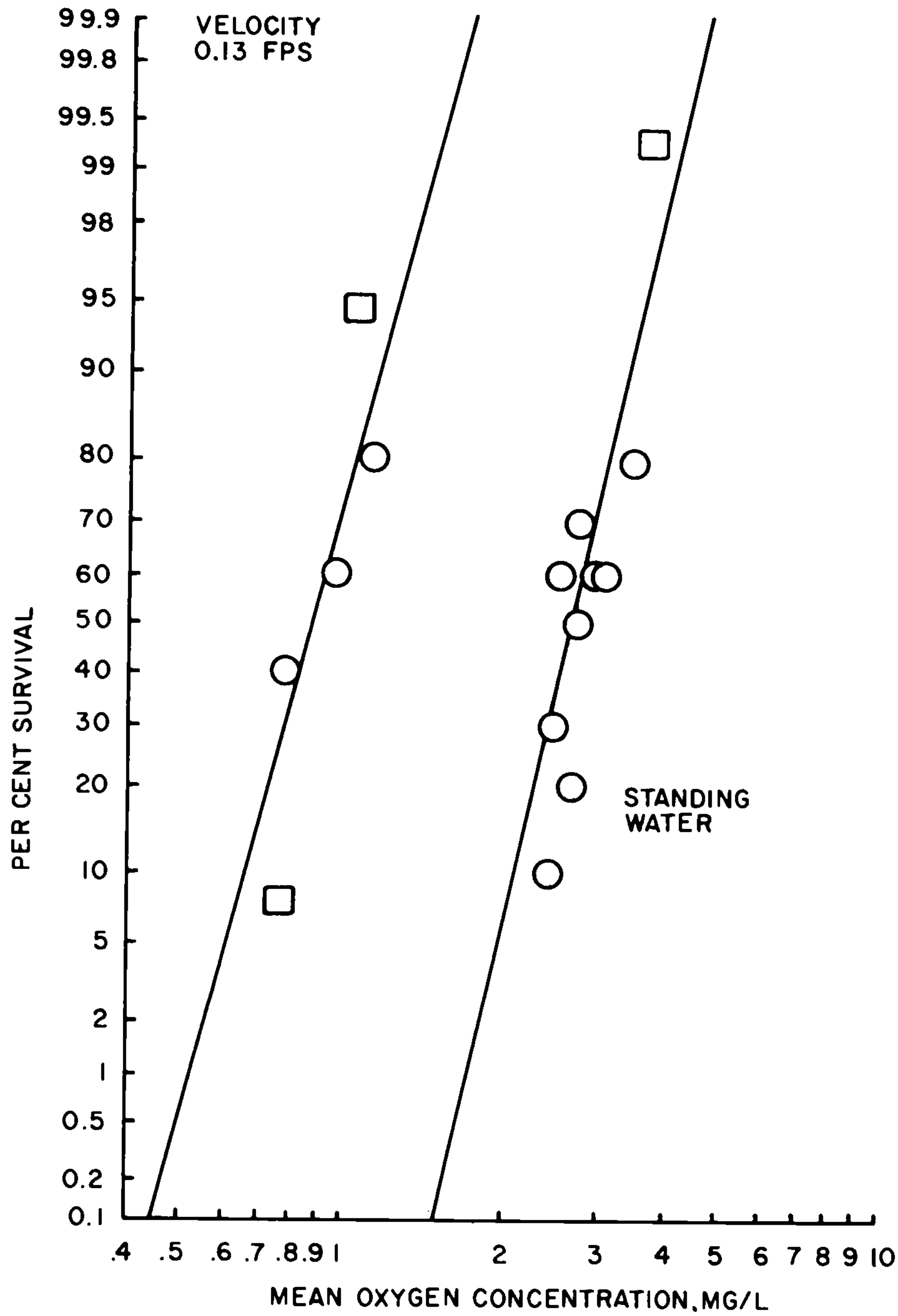

Figure 29. Survival of A. californica (Arcata) in relation to dissolved oxygen, at 2.2°C. in standing water and at a velocity of 0.03 fps.

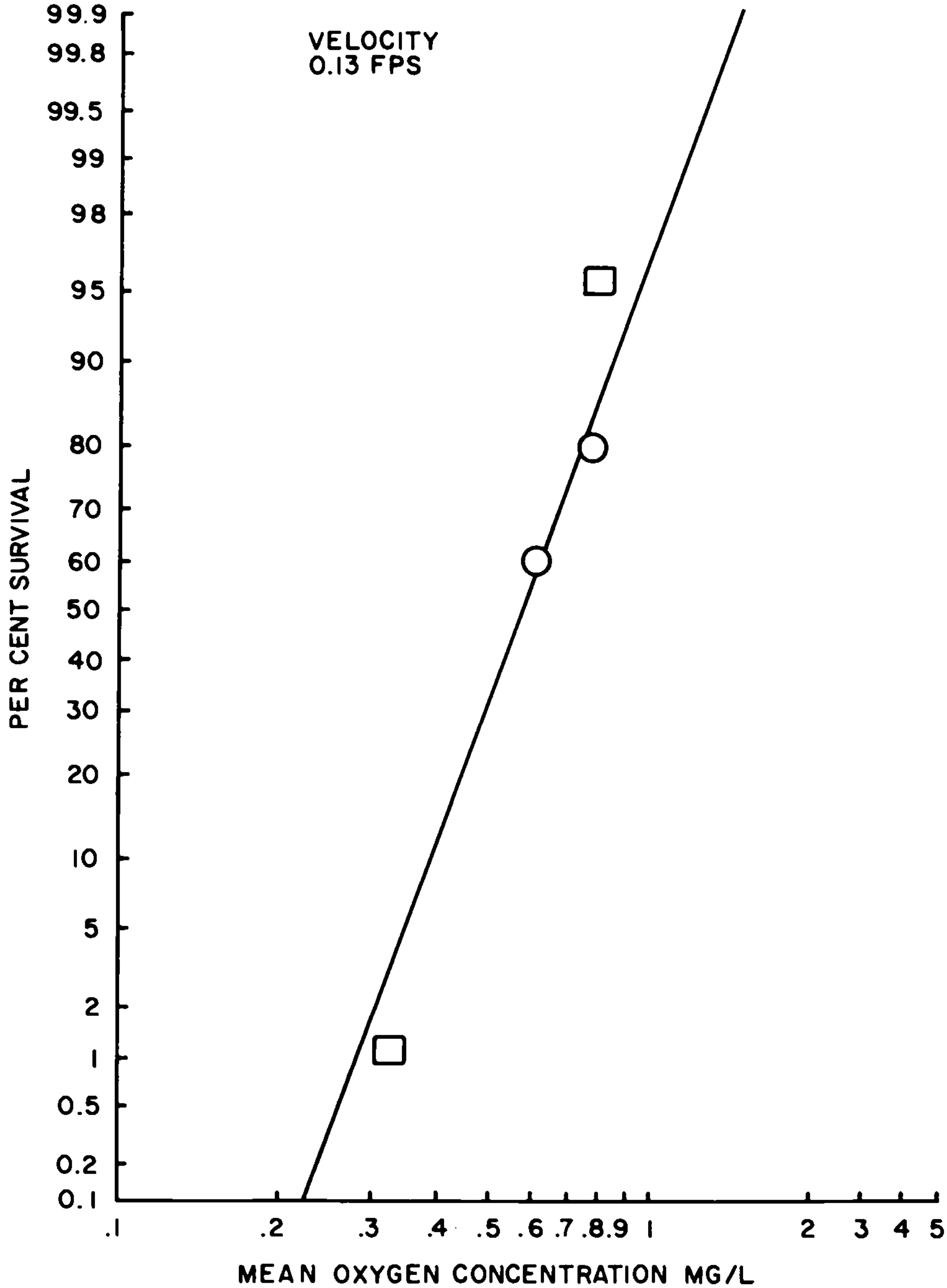

Figure 30. Survival of A. californica (Arcata) in relation to dissolved oxygen, at 2.2°C. and at a water velocity of 0.13 fps.

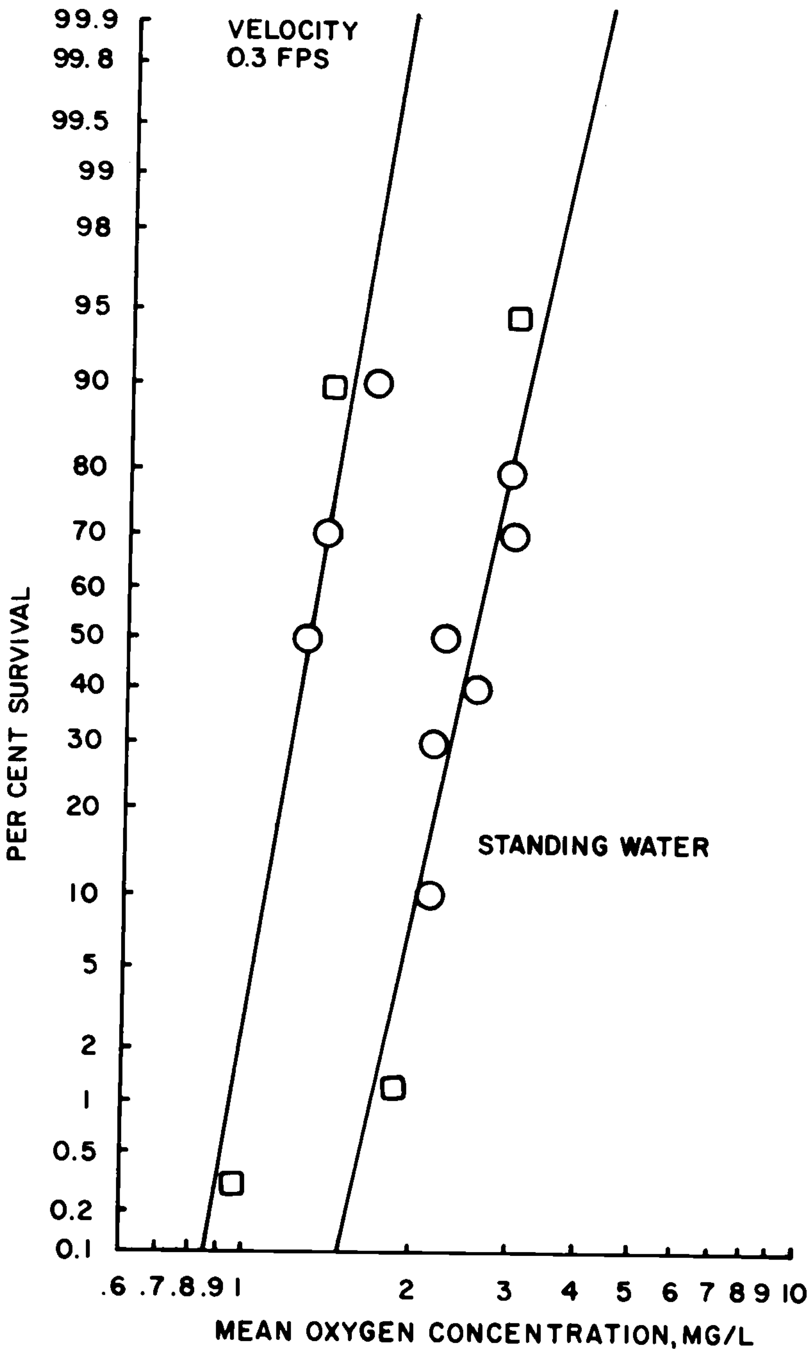

Figure 31. Survival of A. californica (Arcata) in relation to dissolved oxygen, at 14°C. in standing water and at a velocity of 0.03 fps.

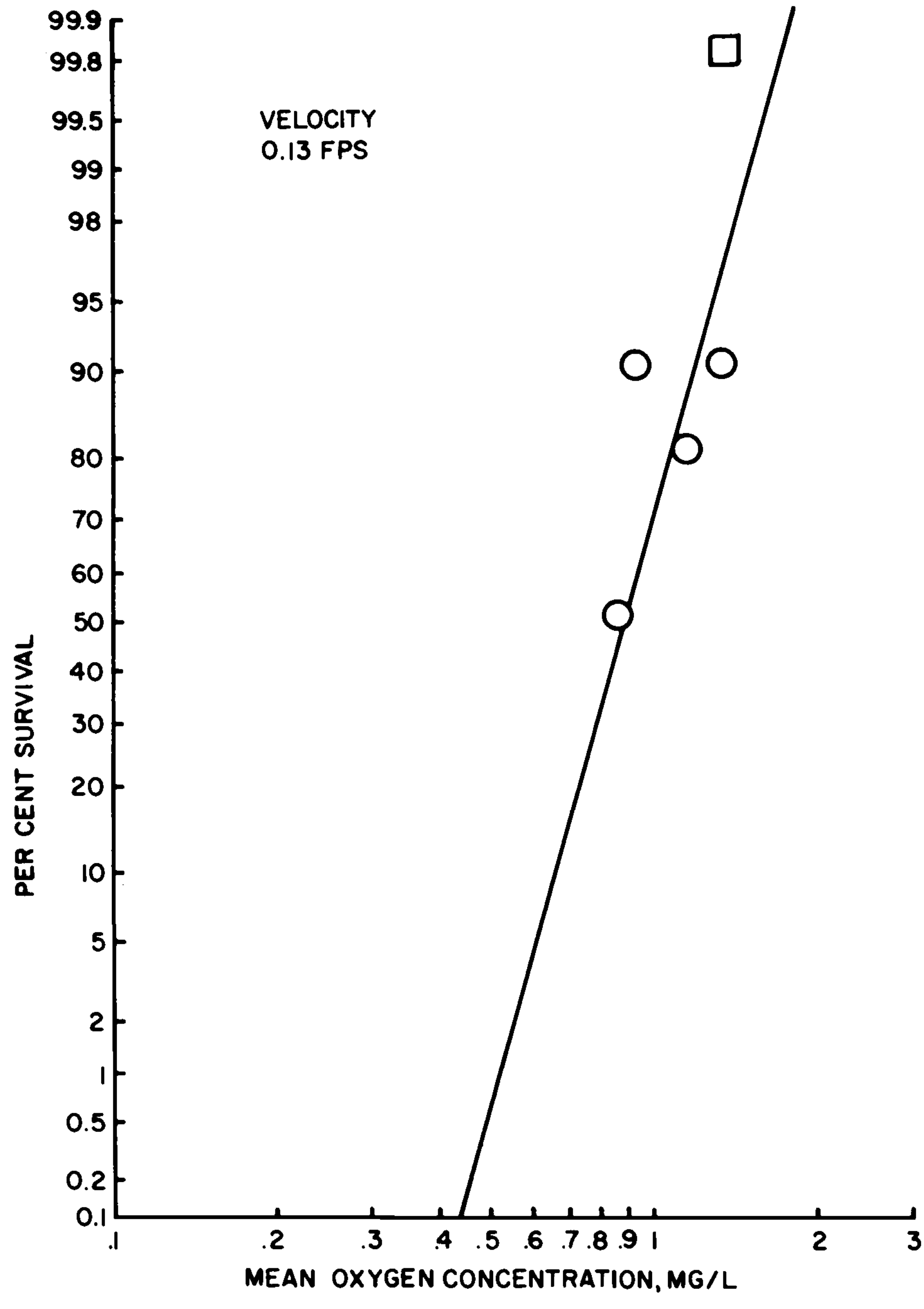

Figure 32. Survival of A. californica (Arcata) in relation to dissolved oxygen, at 14°C. and at a water velocity of 0.13 fps.

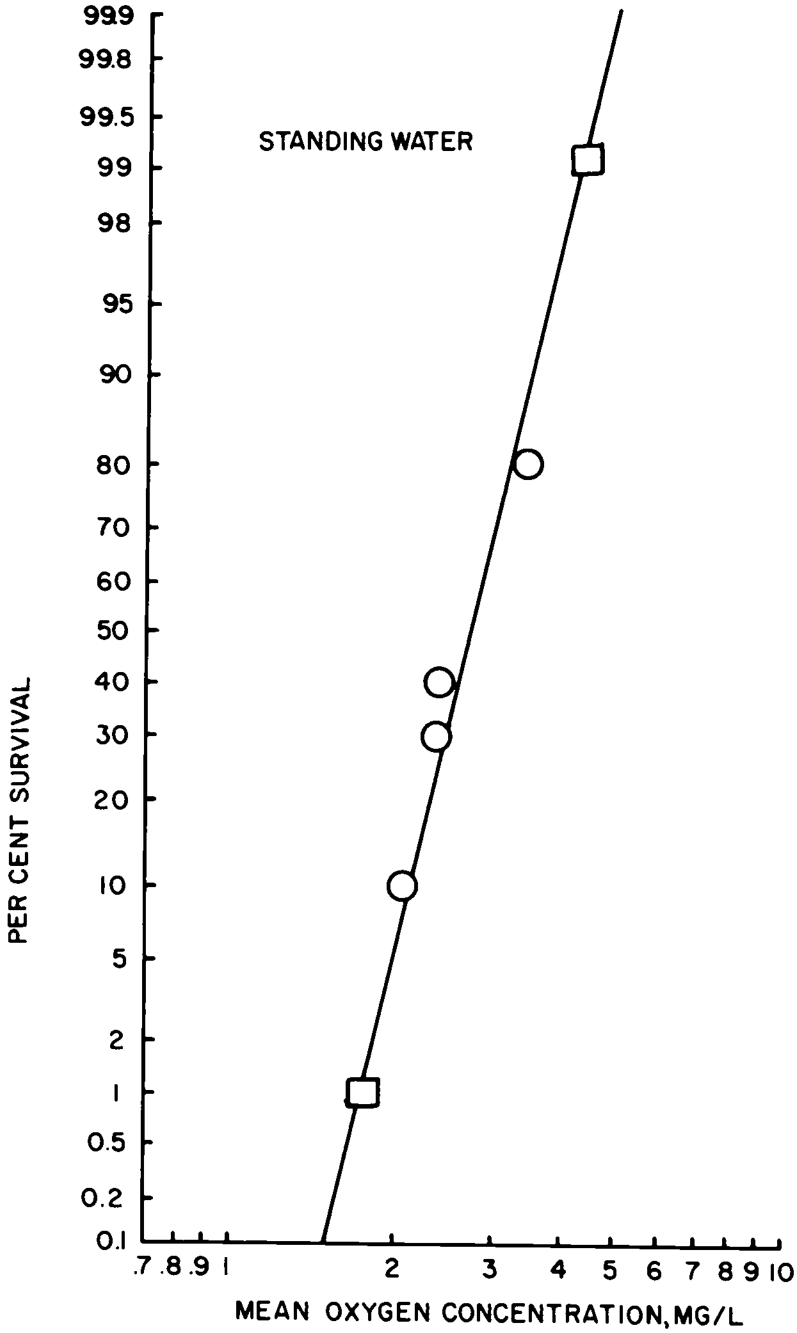

Figure 33. Survival of A. californica (Arcata) in relation to dissolved oxygen, at 20°C. in standing water.

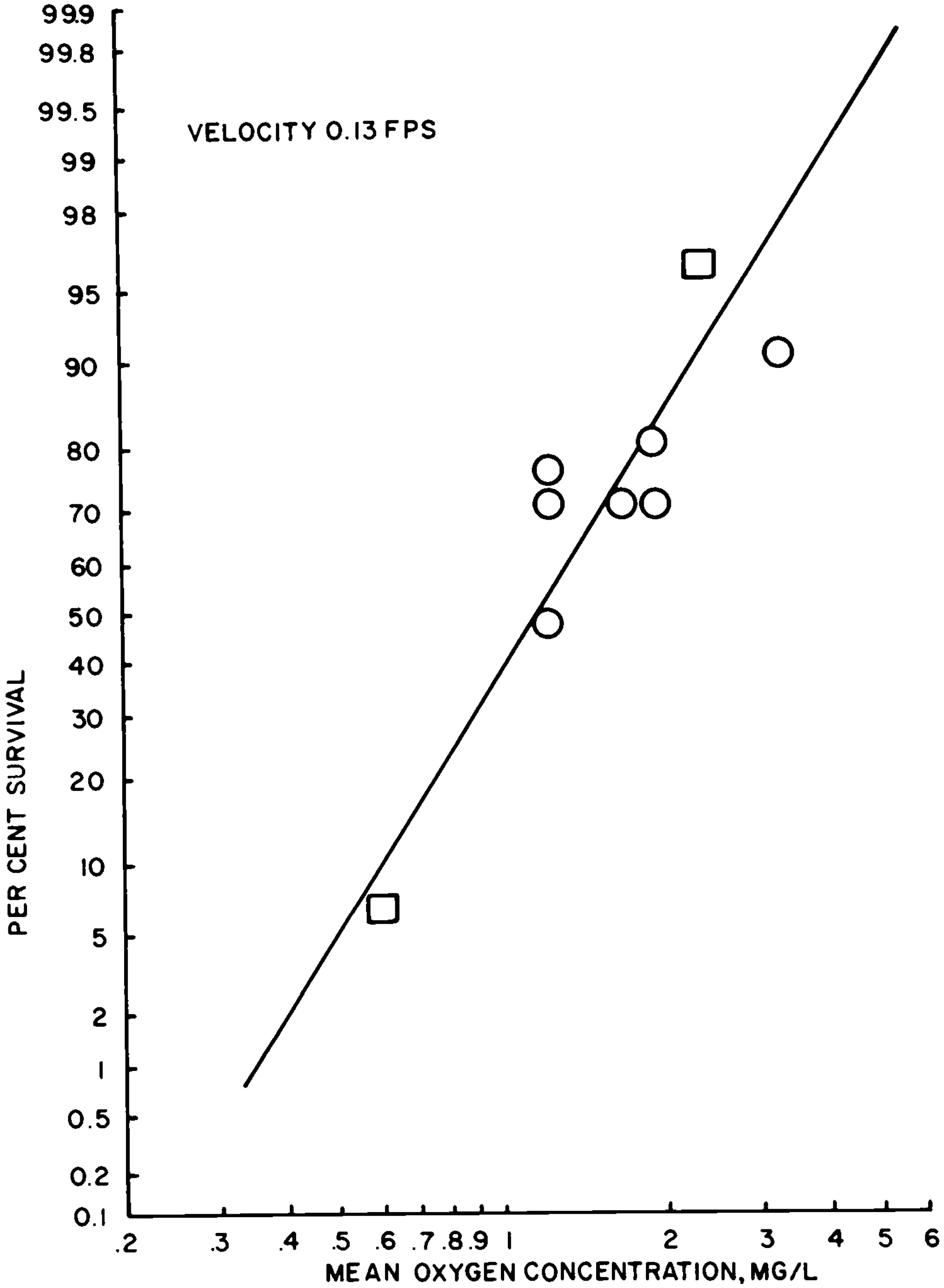

Figure 34. Survival of A. californica (Arcata) in relation to dissolved oxygen, at 20°C. and at a water velocity of 0.13 fps.

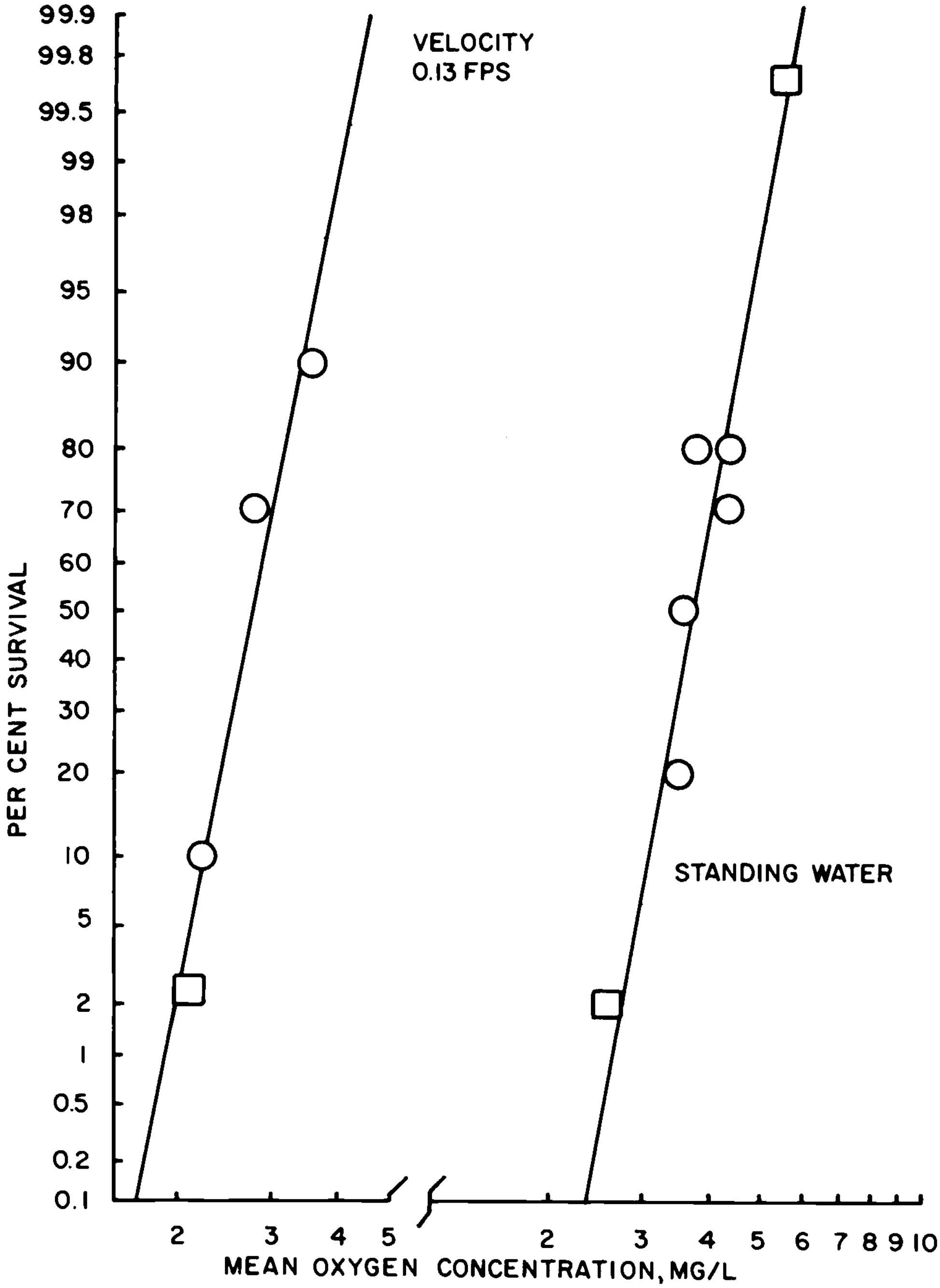

Figure 35. Survival of A. californica (Arcata) in relation to dissolved oxygen, at 24° C. in standing water and at a velocity of 0.03 fps.

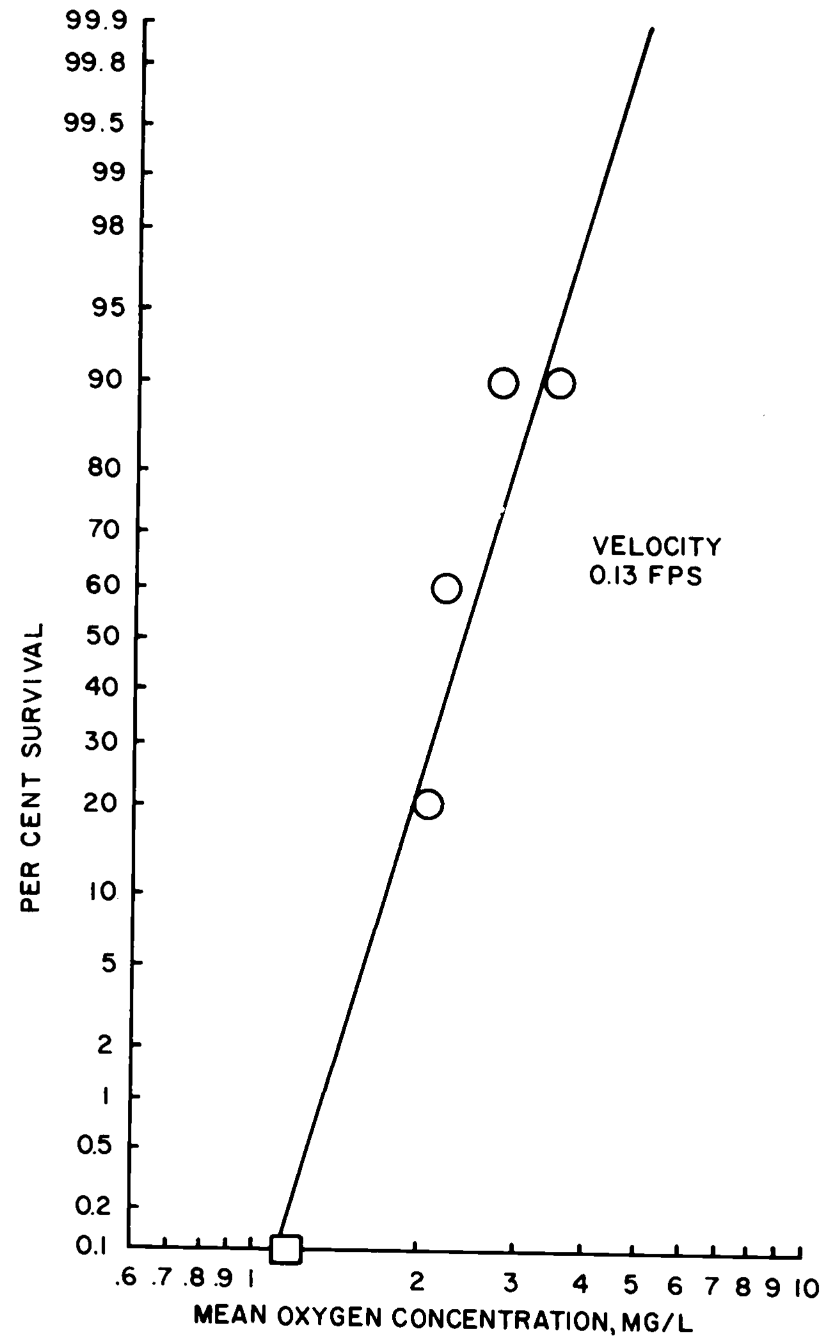

Figure 36. Survival of A. californica (Arcata) in relation to dissolved oxygen. at 24°C. and at a water velocity of 0.13 fps.

metabolic rate and oxygen requirement, but to some other effect of high temperature.

Water velocities from zero to 0.25 feet per second, measured near the center of glass-pipe testing chambers, were tested. The velocities measured and reported herein represented most nearly the maximum velocities existing in the pipes. The selection of a maximum velocity (0.13 f.p.s.) to test regularly was based on the ability of the naiads to maintain a position on the wire screen of the holding capsules in the face of increasing velocities. At velocities above about 0.13 feet per second, naiads were frequently washed off the screen and swept rather helpless by the current to the end of the capsule. Therefore, it seems probable that these insects do not normally live under such conditions of velocity. However, on a "normal" substrate, the naiads may be able to withstand a higher velocity, as measured well away from the substrate, because of the protection which is afforded by Prandtl's "boundary layer" (Ambuhl, 2, p. 138).

The "boundary layer" is a layer of water of varying thickness, depending on the velocity of the freely flowing water and other factors, in which the velocity decreases progressively as the surface of any stationary object exposed to the main current flow is approached.

A difference in the thickness of the "boundary layer" over the screen capsule wall serving as the substrate, could result in TL_m's different from those which would have been determined for the same free flow velocity had the insects been on a solid substrate. In other words, the tolerance to low dissolved oxygen of naiads on solid substrates and on the screen substrate might be different when the free flow velocity is the same. However, if the "boundary layers" over the bodies of the large insects used are more important than these layers over the substrate in influencing tolerance to low oxygen, the tolerance on the two different substrates under otherwise equal conditions should be nearly equal.

Schoenemund (40) believed that the dorso-ventral flattening of the bodies of perlid stonefly naiads is a streamlining adaptation which makes it easier for them to maintain a position in the face of a rapid flow. Ambuhl (2, p. 149-162) confirmed this for some other immature aquatic insects which are able to live in water of high surface velocity because their flattened form allows them to remain within the protection of the "boundary layer" and out of the full force of the flow in the open water. It is also possible that the flattening of Acroneuria naiads is partly to facilitate creeping in the narrow passages between the rocks in a riffle, as they are not normally found above the rocks where they might need protection from relatively high velocities. Stuart

(45, p. 27-35) believes that this flattening among certain mayfly naiads is a "crevice-seeking" rather than a current-resisting adaptation.

Reduced dissolved oxygen concentrations above 3.71 mg/l could be tolerated for 24 hours by at least 50 per cent of the *A. californica* (Arcata) naiads under all the conditions tested. This concentration was the 24-hour TL_m at a temperature of 24°C. in standing water (see Figure 35). However, it is possible that some of the mortality observed at 24°C. was due to the direct effect of high temperature, masking the appearance of the effect due to oxygen deficiency alone.

The lowest oxygen concentration that could be tolerated for 24 hours by 50 per cent of the same test animals under any of the conditions tested was 0.58 mg/l. This concentration was found to be the 24-hour TL_m at a temperature of 2.2°C. and a water velocity of 0.13 f.p.s. (see Figure 30). In this case also it is possible that temperature was directly responsible for some of the effect attributed solely to dissolved oxygen deficiency.

Thus, the point to which dissolved oxygen must be reduced to have a lethal (immobilizing) effect on the average *A. californica* (Arcata) naiad in 24 hours, under the described experimental conditions, is dependent on temperature and velocity and varies accordingly between

0.58 and 3.71 mg/l. Both extremes might be lower for undisturbed naiads in their natural environment and fully acclimatized to the environmental conditions. That acclimatization to reduced dissolved oxygen concentrations occurs in the laboratory is indicated by data on push-up movements to be discussed later.

The data on tolerance and confidence limits for TL_m's are summarized in Table 13. The 24-hour TL_m values presumably represent what the "average" animal can tolerate for a 24-hour period. Thus, the curves in Figure 37 illustrate the tolerance of the average naiad of *A. californica* (Arcata) under all conditions of temperature and velocity tested.

In still water at 2.2°C. the mortality was considerably higher than expected. Even after adjustment for a 50 per cent recovery of naiads adjudged dead initially at 2.2°C., the TL_m is found to be higher at this temperature than at 14°C. An adjusted TL_m was obtained by reducing all mortality values by 50 per cent and using the reduced values to determine the TL_m in the usual manner. Apparently, in still water low temperature is not effective in reducing the lethal effect of reduced dissolved oxygen as it is in flowing water, but on the contrary, it has the opposite effect. Some push-ups do occur at 2.2°C. in still water, but perhaps the low temperature inhibits the

Table 13. Twenty-Four Hour Median Tolerance Limits and Confidence Limits, in mg/l of Dissolved Oxygen, of Stonefly Naiads.[1]

Test Animal	Temp. °C.	Water Velocity in Feet Per Second											
		0.00		0.03		0.06		0.13		0.17		0.25	
		TL_m	C.L.	TL_m	C.L.	TL_m	C.L.	TL_m	C.L.	TL_m	C.L.	TL_m	C.L.
Acroneuria californica (Corvallis)	20	-	-	-	-	2.33	2.16-2.51	-	-	1.59	1.48-1.70	1.85	1.57-2.17
Acroneuria pacifica (Corvallis)	20	-	-	2.85	2.55-3.18	-	-	-	-	-	-	-	-
Acroneuria californica (Arcata)	2.2	2.66	2.51-2.82	0.87	0.79-0.96	-	-	0.58	0.44-0.76	-	-	-	-
do	14	2.54	2.39-2.70	1.26	1.18-1.35	-	-	0.89	0.77-1.03	-	-	-	-
do	20	2.70	2.39-3.05	-	-	-	-	1.20	1.00-1.43	-	-	-	-
do	24	3.71	3.53 3.90	2.73	2.37 3.14	-	-	2.34	2.06-2.66	-	-	-	-

1 Confidence limits of TL_m's at the 95% level of probability.

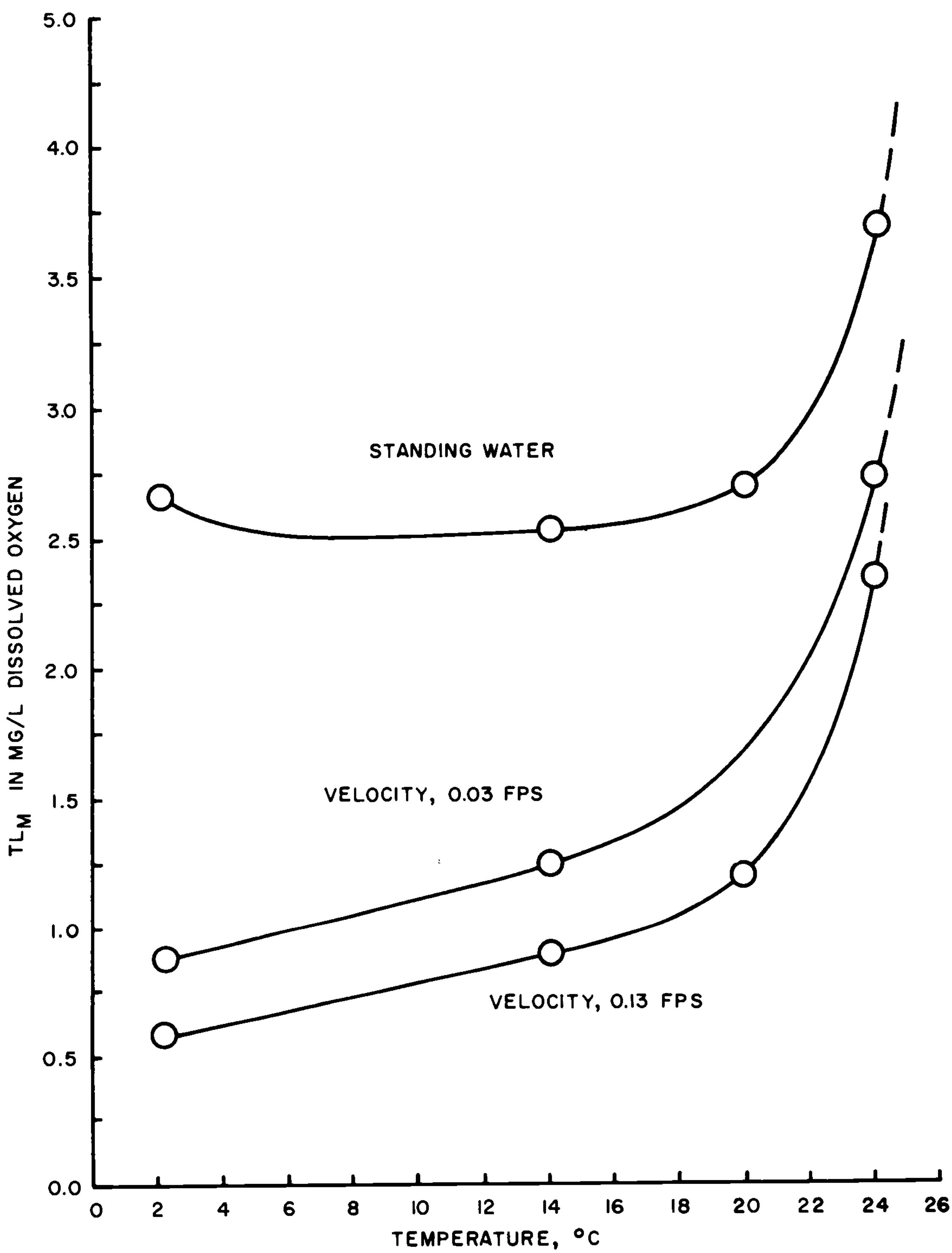

Figure 37. Median tolerance limits of reduced dissolved oxygen for A. californica (Arcata) in relation to temperature, at different velocities.

vigor of push-ups or reduces their rate, thus interfering with the uptake of dissolved oxygen. In flowing water, the movement of the water may render ventilatory movements relatively unimportant at very low temperatures.

The low oxygen tolerance of naiads of A. californica (Arcata) is compared with that of A. californica (Corvallis) and A. pacifica (Corvallis) in Figure 38. The observed differences in the tolerance between naiads of A. californica from the two areas are suprisingly great. However, as the data on the naiads from the Corvallis area are few, and as the experiments with the two groups were not nearly simultaneous, no sweeping conclusions are drawn. The comparison of the tolerance of A. californica naiads from four or five ecologically different geographic areas could be the subject of a very interesting investigation. It appears that the differences between representatives of the same species from different localities may sometimes be greater than the differences between different, but closely related species from the same waters.

Figure 38 shows that an increase in the velocity of the flow beyond 0.13 f.p.s. (about 4 cm./second) can have relatively little depressing effect on the TL_m of dissolved oxygen. Ambuhl (2, p. 243) noted in his work with the larvae of the caddisfly, Hydropsyche angustipennis, that when a certain velocity (a few centimeters per second)

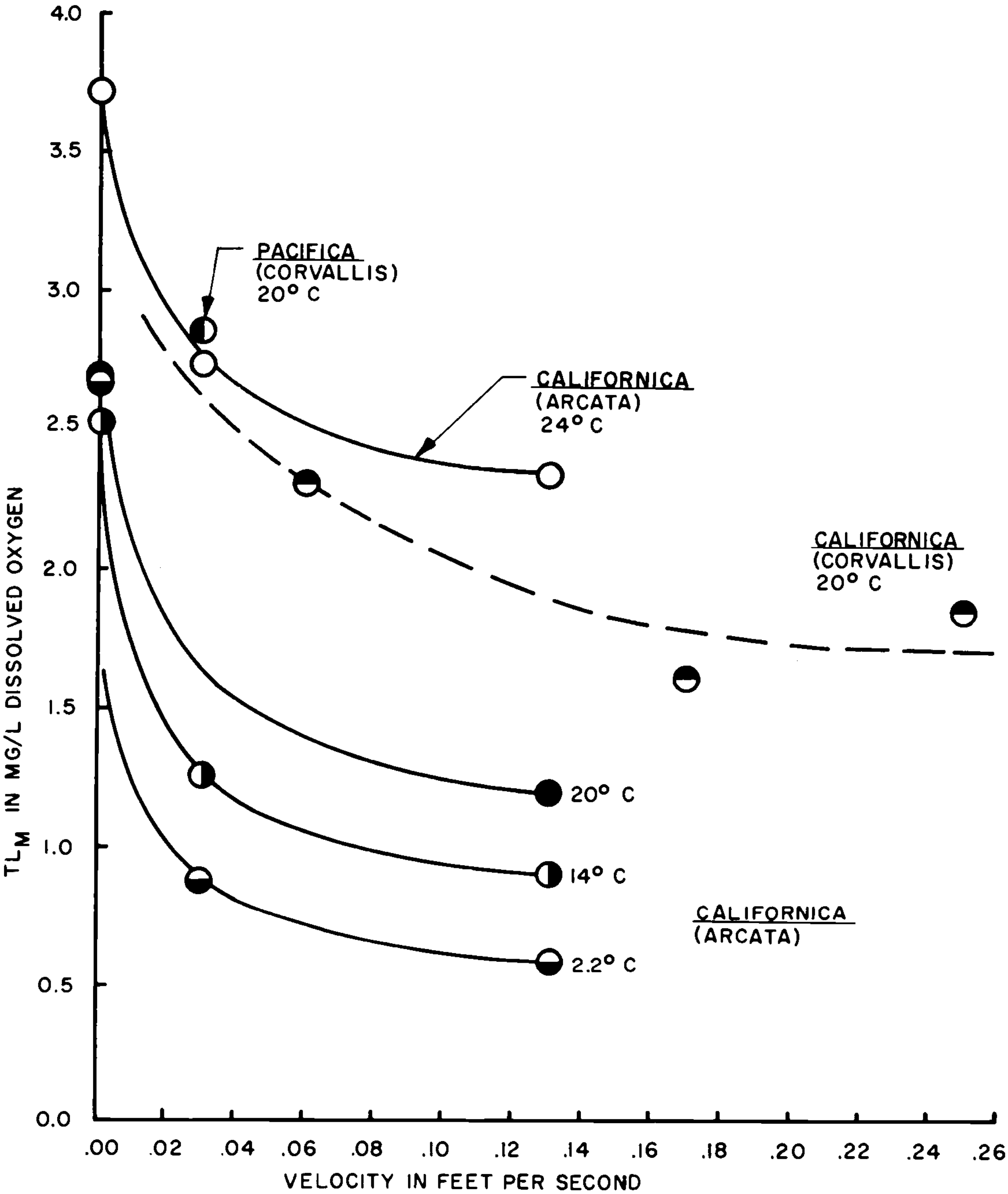

Figure 38. Median tolerance limits of dissolved oxygen for *Acroneuria* from different localities in relation to water velocity, at different temperatures.

was reached, a further increase in velocity did not cause the lethal oxygen concentration to drop further. Ambuhl interprets his findings to mean that when the velocity of the free water flow is increased to the "saturation point," the thickness of the "boundary layer" over the body of the animal is so reduced that the layer no longer effectively impedes the diffusion of oxygen from the freely flowing water to the organism.

Actually, an effective "boundary layer" does not, of course, obstruct diffusion of oxygen. However, the rate of delivery of oxygen to the animal's respiratory surfaces must increase as the velocity of the freely flowing water increases to the "saturation point," causing an increase also of the velocity of water in the immediate vicinity of the respiratory surfaces. At the "saturation" point, the velocity must be such that any further increase thereof beyond this level cannot materially increase the rate of oxygen delivery, or of displacement of partially deoxygenated water from the immediate vicinity of the respiratory surfaces.

Several long-term recirculation experiments were carried out to investigate how well the results of 24-hour tests represent the longer term tolerance of the naiads to low oxygen. These experiments were performed with _A. californica_ (Corvallis) only. In general, under a given set of conditions, there apparently was not much, if any, additional mortality due to low oxygen after the first 24 hours of the effective test period. In one experiment where the oxygen concentration was maintained at a maximum of about 3.4 mg/l (mean, 3.06, and range 2.7 to 3.4 mg/l) for 12 days, there was no mortality among 10 naiads during the first two days. On the third day one naiad was dead and by the twelfth day four were dead. One of these was observed being killed and devoured by one of the other naiads, and it appeared that the other three deaths could have been similarly violent. In 24-hour tests with A. californica (Corvallis) under the same conditions, the TL_m was 2.33 mg/l and the point at which lowered oxygen was just non-lethal was about 3.0 mg/l. At relatively high oxygen levels (where there is no great physiological stress due to insufficient oxygen), cannibalism could be expected to increase mortality as the time period increases. However, observations made during the tube experiments show that at oxygen levels where there is stress, cannibalism is reduced or absent, depending on the degree of stress and preoccupation of the naiads with ventilatory activity. Thus, at oxygen concentrations that result in the death of one or more of the test animals, the figures for mortality may be either less influenced or not at all influenced by cannibalism.

The mortality at any given oxygen concentration is dependent on water temperature and on water velocity, and one of these factors is perhaps generally as important as the other in its influence on mortality caused by a oxygen deficiency, within the range of conditions studied. Sufficient information on naiads of A. californica (Arcata) is presented to make it possible to estimate whether reduction of the oxygen concentration to any given level would or would not by itself cause death of A. californica (Arcata) at any given combination of water temperature and velocity within the ranges studied.

As indicated already, both the rates of ventilatory movements, herein referred to as "push-ups," and the 5-minute total number of these movements have been determined and shown to be related to oxygen concentration, to temperature and to water velocity. The rates of these movements are different in the two species of Acroneuria studied. They indicate some of the environmental conditions to which individuals are exposed, and some physiological adjustments being made.

At relatively high dissolved oxygen levels, if push-ups occur at all, the relationship between dissolved oxygen and the push-up rate is not clear. Frequently, at such high concentrations of oxygen, and especially just after the animals were disturbed, the intervals between series of push-ups were very irregular and the push-ups generally occurred in short "bursts" lasting one to five seconds each.

The rates of those individuals which were performing push-ups continuously over a period of 20 seconds or more are the principal ones considered in this report. Only a regular and more or less sustained rate of push-up movements was believed to reflect the existing conditions of dissolved oxygen, although as stated, the total number of push-ups over a 5-minute period is also related to the oxygen concentration.

The counting period of 20 seconds was settled on after 10, 15, 20, and 30-second counts for six concentrations of dissolved oxygen were compared by "analysis of variance" (Snedecor, 42, p. 283-299). The basic experimental data, obtained in an experiment in which the dissolved oxygen was lowered stepwise over a period of six hours, are presented in Table 14.

Push-ups commonly occurred in bursts and it seemed possible that the push-up rate could be different at different points of time during a single burst. Toward the end of a burst, for example, the rate might be expected to decrease as the ventilatory need becomes satisfied, and a rate determination made from counts during a short time period covering the first half of a burst might yield a higher value than a determination made over a longer time period covering all or most of a burst. However, it was found that the interaction of length of the counting period and the dissolved oxygen level was not significantly greater than zero.

The differences between push-up rates at the various concentrations of dissolved oxygen and for the various counting intervals were highly significant, at a probability level of 0.01. Certain differences between subclass means (for 5.54 mg/l, dissolved oxygen, and time periods of 15 and 20 seconds; 6.15 mg/l, 10 and 15 seconds; 7.45 mg/l, 10 and 15 seconds) were inconsistent due to the unequal numbers of observations on push-ups by naiads whose push-up rates differed considerably from those of other naiads. The general difference between the rates for periods of 15 and 20 seconds was not significantly greater than zero, at the 0.05 level of probability. There was a significant general difference between the rates for periods of 10 and 15 seconds and 20 and 30 seconds. In each of these pairs the longer time period is by 50 per cent greater than the shorter, whereas the 20 second period is greater than the 15 second period by only 33-1/3 per cent.

As Table 14 shows, means of the mean push-up rates are lower for longer time periods. This probably means that the longer periods give more representative counts, the error in counting being smaller over a longer time. In spite of this it was desirable to select the 20-second period over the 30-second period for routine counting because considerably fewer 30-second counts could have been made, particularly at the higher concentrations of oxygen. That is, the number of naiads performing push-ups continuously for at least 20 seconds is greater than the number doing so for 30 seconds or more at the higher concentrations of dissolved oxygen, and obtaining sufficient counts was sometimes a problem.

Figure 39 shows the relationship between push-up rate for A. californica (Arcata), water velocity, and temperature as determined in experiments not exceeding 24-hours in duration. It will be seen that at each combination of temperature and water velocity tested, the push-up rate was highest at some oxygen concentration which varied with the temperature and the velocity. At progressively lower oxygen concentrations, the push-up rate declined rather sharply, and at progressively higher concentrations it also declined, but less sharply, apparently to a constant level.

A marked effect of temperature on the relative positions of the curves relating mean push-up rates to oxygen concentration is evident. It is apparent that

Table 14. Summary of Data on Push-Up Rates of *A. californica* (Arcata) and Dissolved Oxygen (in Standing Water at 24°C.) used in Analysis of Variance.[1]

Time in Sec.		Concentration of Dissolved Oxygen in mg/l 2.40	3.36	4.35	5.54	6.15	7.45	Totals
10	Mean No.	51.36	54.67	55.91	58.18	60.00	58.67	55.95
	n[2]	25	30	23	22	16	15	131
15	Mean No.	49.59	52.42	53.96	56.73	60.20	58.79	54.44
	n	21	26	21	20	13	10	111
20	Mean No.	49.24	51.75	53.40	57.31	58.22	57.71	53.49
	n	21	24	20	16	9	7	97
30	Mean No.	48.80	49.44	50.19	53.09	56.23	55.50	51.07
	n	15	20	15	13	6	5	74
Totals	Mean No.	48.89	52.34	53.67	56.65	59.18	58.09	54.09
	n	82	100	79	71	44	37	413

1 Push-up values are the numbers of push-ups for the 20-second counting periods, or, for other counting periods, the equivalent number per 20-second period. All counts were made during the last 20 minutes of 1-hour periods during which a constant oxygen concentration was maintained.

2 Number of individual observations. All counts were made during separate timings.

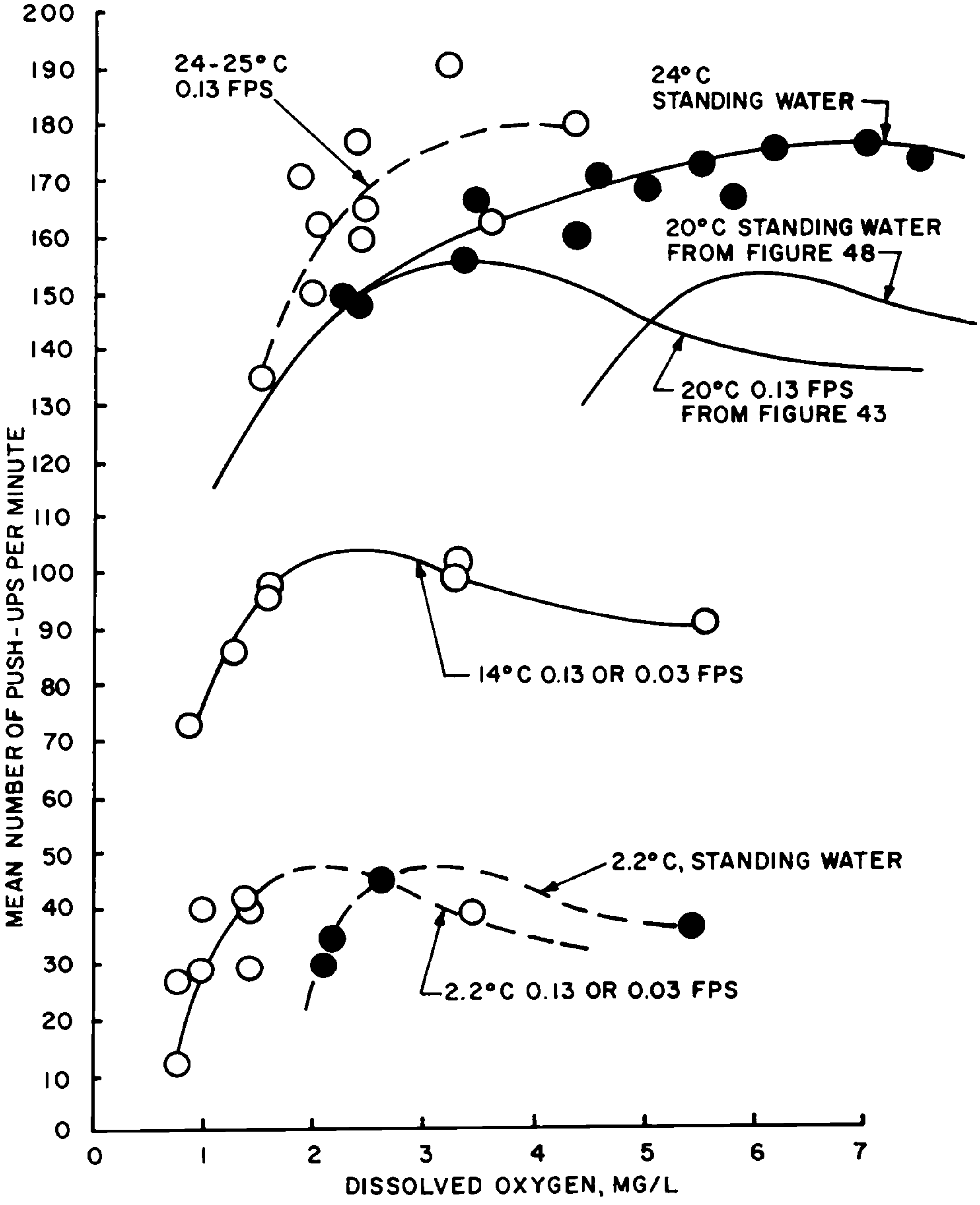

Figure 39. Relationship between push-up rates for A. californica (Arcata) and dissolved oxygen at different temperatures. Data from experiments of 24 hours or less duration.

temperature acts as a controlling factor regulating push-up rates more or less independently of the ventilatory requirements of the animals. At a water velocity of 0.13 f.p.s., an increase in temperature from 2.2°C. to 14°C. (a change of 11.8°C.) increases the maximum rate of push-ups by about 100 per cent. An increase from 14°C. to 24° -25°C. (a change of 10 or 11°C.) also increases the maximum push-up rate by about 100 per cent (see Figure 39).

It will be noted that the peaks of the two push-up rate curves obtained at 24-25°C. for two widely different water velocities (0 and 0.13 f.p.s.) are virtually at the same level. The same is true of the peaks of the two 20°C. curves. With respect to the dissolved oxygen concentrations at which the maximum push-up rates were observed, there is a great difference between the two curves obtained with and without current at each temperature level.

It can be seen in Figure 39 that at any given temperature (e.g., 20°C.), the push-up rate of naiads in standing water may be greater than, equal to, or less than the rate in flowing water, depending on the oxygen concentration. The influence of velocity on the push-up rate thus is variable, and under some conditions, little or no effect of velocity changes is to be expected. This conclusion is confirmed by data in Table 15, which shows the influence of water velocity on the push-up rates and also on the "weighted push-up rates" observed in two experiments performed at different temperatures and oxygen concentrations. At 14-15°C. and dissolved oxygen concentrations of 6.35-6.78 mg/l, the push-up rate decreased with increasing velocity. At 20°C. and oxygen concentrations of 3.83-3.88 mg/l, velocity had very little effect on the push-up rate, and the highest rate was observed at the highest velocity. The weighted push-up rates in Table 15 show, on the other hand, a consistent, progressive decline with increasing velocity under both sets of experimental conditions. Evidently these rates reflect the ventilatory requirements of the animals better than do the unweighted push-up rates which may even decrease when the total ventilatory activity has been increasing through increase in the frequency and duration of the intermittent "bursts" of such activity.

In Figures 40 and 41, the weighted push-up rates obtained in the two experiments (Table 15) are plotted against water velocity. In one experiment (Figure 40) the velocity was increased from zero to 0.250 f.p.s. in hourly increments over a 6-hour period. In the second (Figure 41) the velocity was increased in five steps, then decreased in five as a check on whether elapsed time or the direction of change in velocity had any effect on the push-up rates. As Figure 41 shows, these factors had little or no effect. The curves in Figures 40 and 41 show that the weighted push-up rates decrease as water velocity increases to about 0.2 f.p.s., at least.

While it appears that the low oxygen tolerance of naiads of A. pacifica (Corvallis) is not greatly different from that of naiads of A. californica (Corvallis), the difference in their push-up rates at any given concentration of oxygen and at the same temperature and similar velocities is striking (Figure 42). As Figure 43 shows, the rates of A. californica (Arcata) are likewise different from those of A. pacifica (Corvallis). In fact, there is virtually no overlap in either mean or individual rates. Differences in push-up rates demonstrate a physiological difference between these two species that are morphologically and otherwise quite similar and are frequently found together on the same riffle. In spite of the rather surprising observed differences in low oxygen tolerance between A. californica naiads from Arcata and Corvallis, their push-up rates seem to be very nearly alike as far as can be determined from the few observations made.

Table 15. The Effect of Stepwise Changes in Velocity on Push-Ups of Individually Marked Naiads of A. californica (Arcata).

Exp.	End of Hour	Veloc. f.p.s.	Mean Push-up Rate[1]	Per Cent of Naiads Performing Push-ups[2]	Weighted Push-up Rate[3]	No. of Animals	Tempera-ture °C.	Dissolved Oxygen mg/l
1 4/2/60	1	0.000	138.0	100	138.0	10	14-15	6.35-6.78
	2	0.017	138.0	80	110.4			
	3	0.033	122.1	50	61.1			
	4	0.067	113.7	40	45.5			
	5	0.125	114.9	10	11.5			
	6	0.250	-	0	0.0			
2 7/21/60	1	0.000	151.5	100	151.5	20	20	3.83-3.88
	2	0.017	146.7	75	110.0			
	3	0.033	147.2	65	95.7			
	4	0.067	151.5	50	75.8			
	5	0.125	142.5	20	28.5			
	6	0.250	156.0	10	15.6			
	7	0.125	155.1	15	23.3			
	8	0.067	153.0	25	38.3			
	9	0.033	151.5	65	98.5			
	10	0.017	151.8	80	121.4			
	11	0.000	148.8	100	148.8			

1 Push-ups per minute calculated from 20-second counts and based only on those naiads performing push-ups.

2 Regularly for 20-second periods during the last 20 minutes of each hour except for hour No. 2 of the first experiment, in which the observations were made during the first 20 minutes.

3 Mean push-up rate multiplied by the estimated fraction of test animals performing push-ups at any one time.

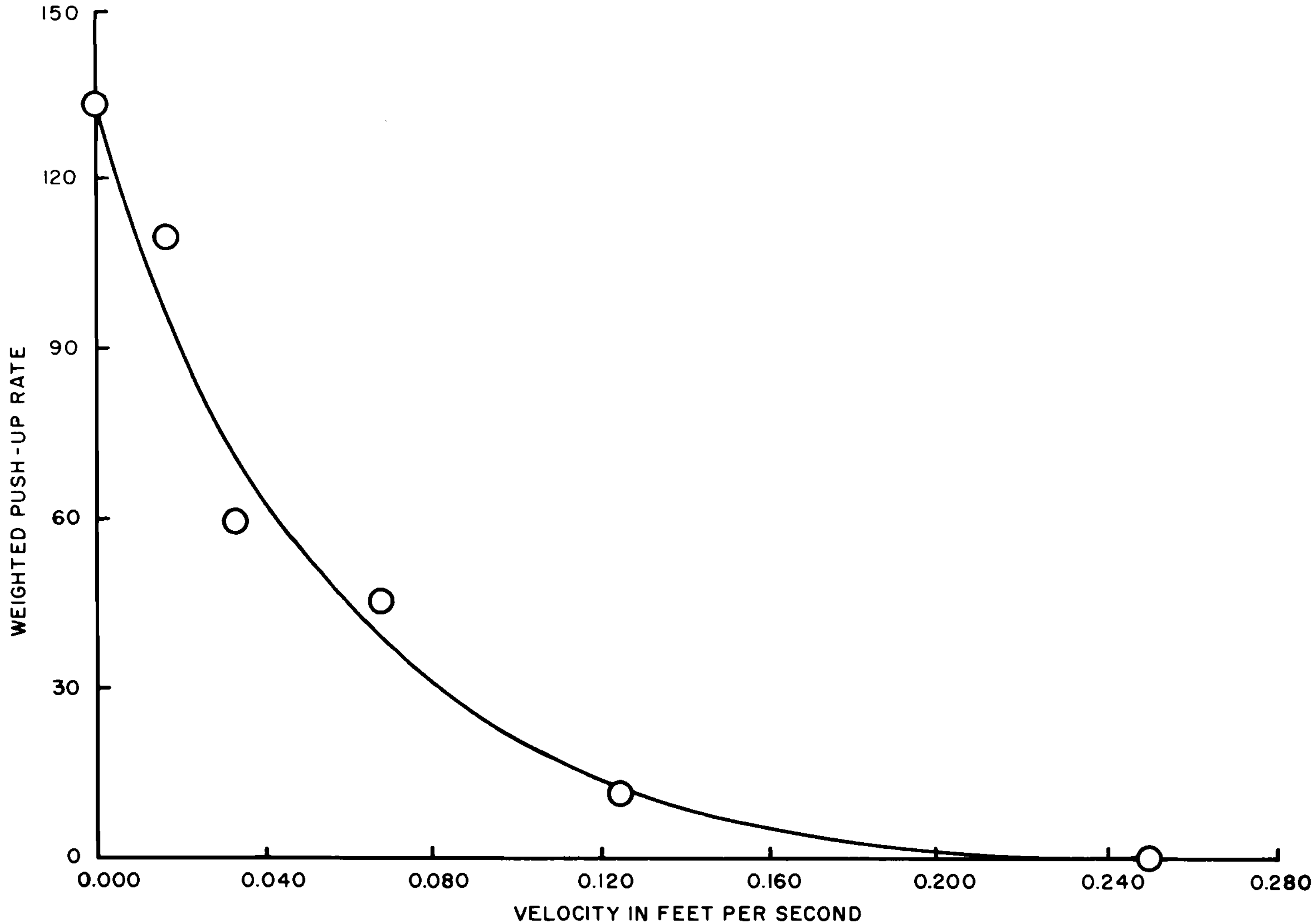

Figure 40. The effect of water velocity on weighted rate of push-ups of A. californica (Arcata) at dissolved oxygen concentrations of 6.35 to 6.78 mg/l and 14-15°C. Data from Table 15.

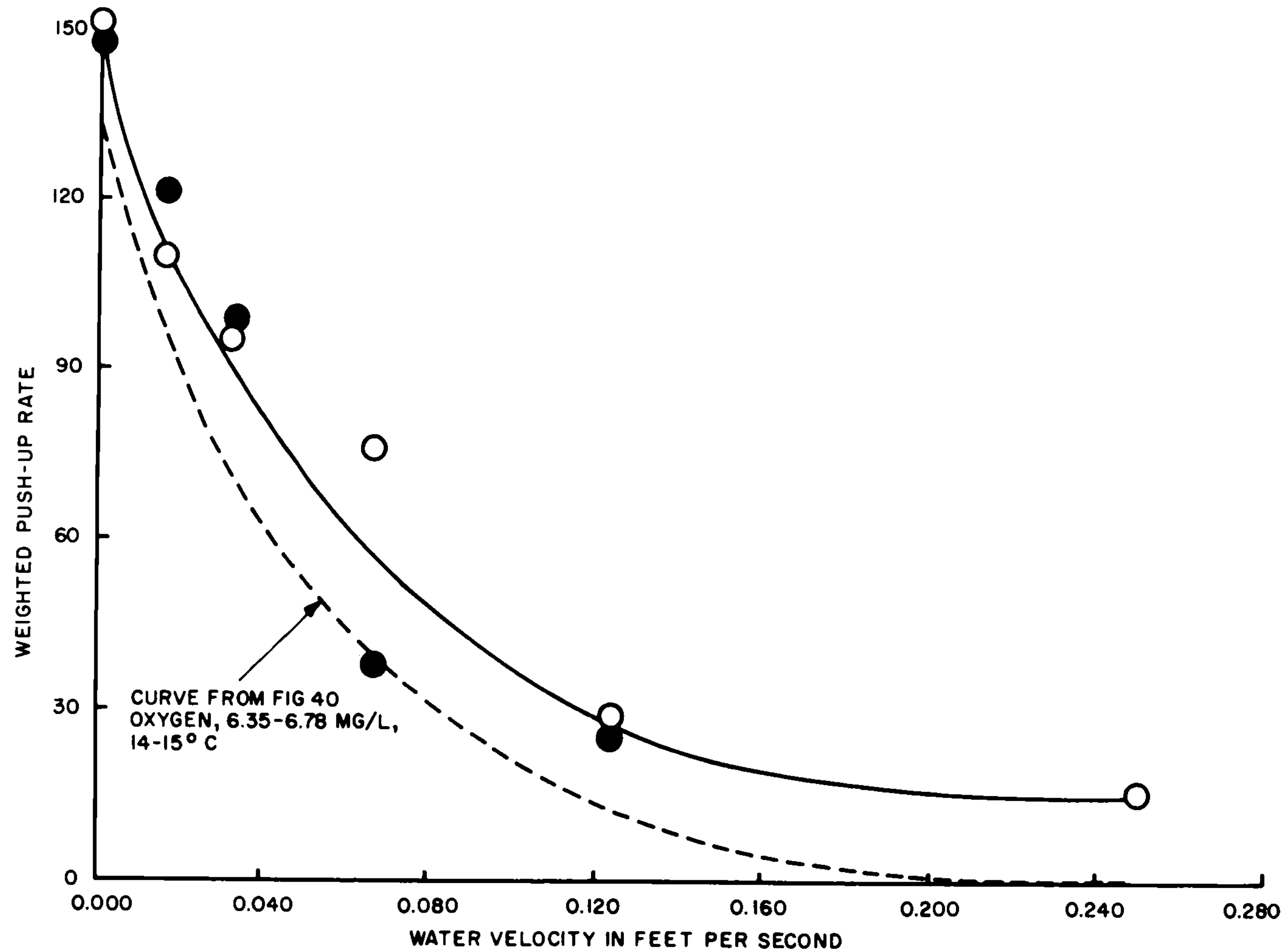

Figure 41. Weighted push-up rate for A. californica (Arcata) at dissolved oxygen concentrations of 3.83-3.88 mg/l and at 20°C. in relation to water velocity. Chronological order of the observations is from left to right for the unshaded circles and from right to left for the shaded circles. Data from Table 15.

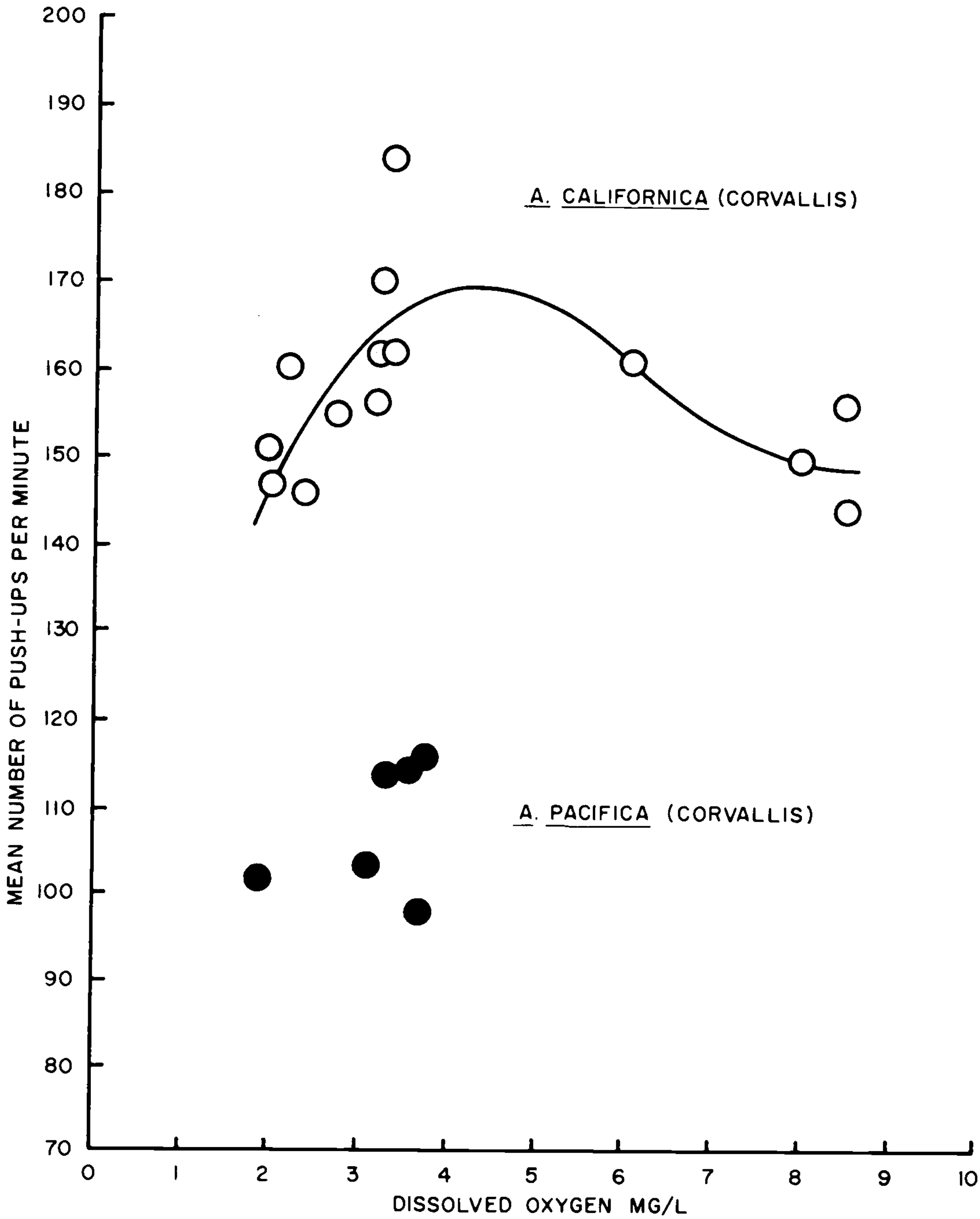

Figure 42. Push-up rates for Acroneuria in 24-hr. tube experiments at 20°C. and water velocity of 0.06 fps.

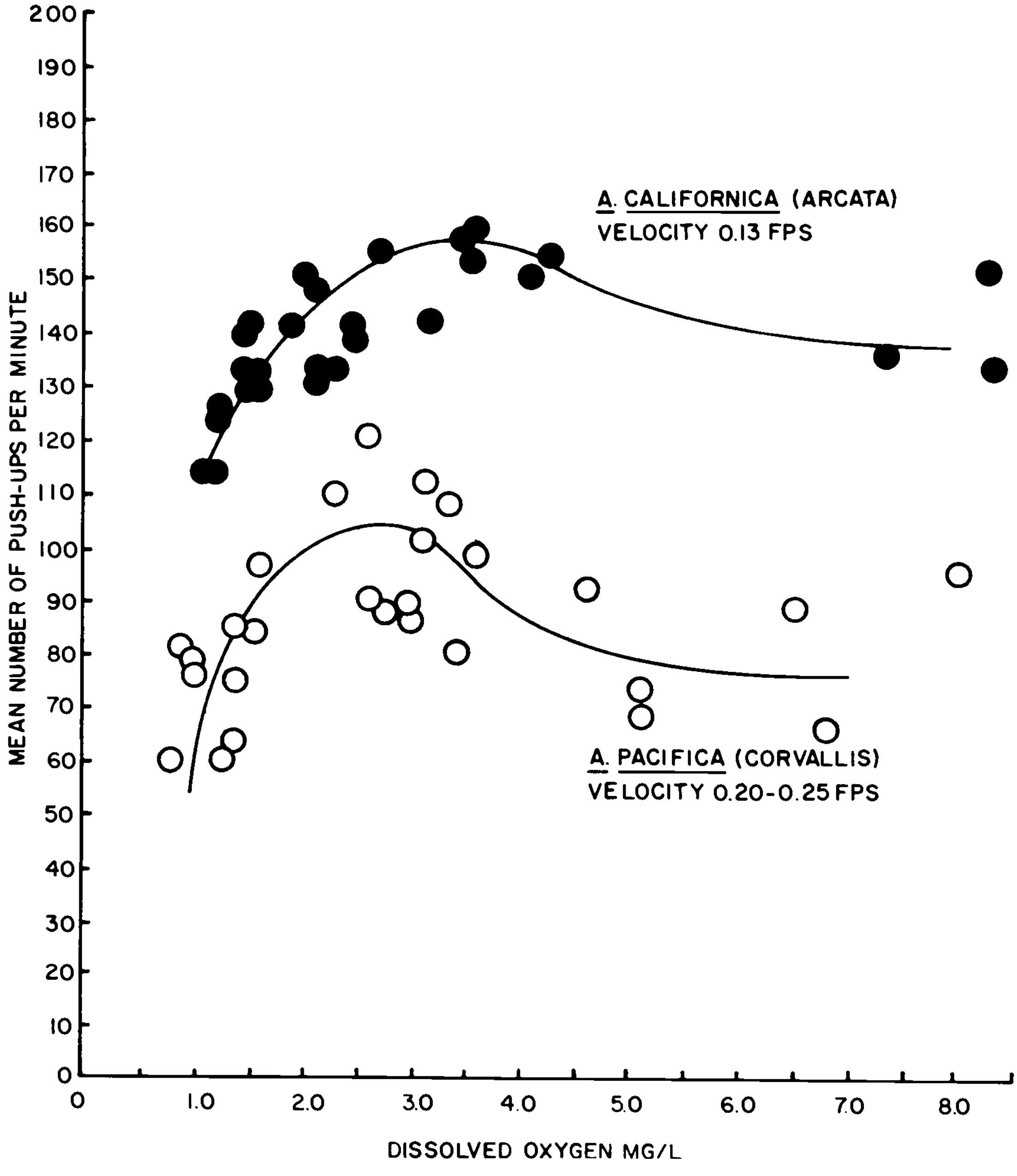

Figure 43. Push-up rates for A. californica (Arcata) and A. pacifica (Corvallis) in relation to dissolved oxygen at 20 C. and at high velocities. Data from experiments of 24 hours or less duration.

Ventilation movements similar to push-ups are characteristic of quite an array of immature aquatic insects and have often been the subject of very general investigation.

A relationship has been reported between rates of ventilation movements and the concentration of dissolved oxygen in the mayfly *Cloeon dipterum* (Wingfield, 54) and in two species of case-caddisfly larvae (Van Dam, 48; Fox and Sidney, 17, p. 325), the rate being higher at lower concentrations, up to a point, at least. Philipson (36) found a relationship between the rate of the abdominal movements of the larvae of the caseless caddisfly, *Hydropsyche instabilis*, dissolved oxygen concentration and water movement which was not unlike that reported here for push-ups in *Acroneuria*.

Ambuhl (2, p. 243-245, 251, 252) counted the ventilation movements of two species of caddisfly larvae at different concentrations of dissolved oxygen. He plotted curves relating ventilation movements of *Hydropsyche angustipennis* to oxygen concentration. These curves are somewhat similar in shape to those obtained with *Acroneuria*. He apparently considered the oxygen concentrations at which the peaks of these curves occurred to be the same as the lethal threshold concentrations. However, as with *Acroneuria* (Figure 44, this thesis) these peaks are at considerably higher oxygen concentrations than those shown to be lethal to more than half of the test animals. Philipson's (36) data show about the same thing for Hydropsyche instabilis. The 5-minute total counts of push-ups, like the weighted push-up rates, probably are more indicative of the ventilatory needs of the naiads than are the 1-minute unweighted rates. That is, the 5-minute total counts probably indicate, better than the 1-minute rates, the general respiratory difficulty (due to inadequate dissolved oxygen or water velocity) that a particular naiad is in.

Figure 44 shows the relation between 5-minute total counts and oxygen concentration in standing water at 20 and 24°C. It was not possible to plot on this graph some very low counts that were obtained at 20°C. when the oxygen concentration was near the air-saturation level (about 9 mg/l), but the 20°C. curve has been drawn to conform with these observations. As shown in Figure 44, the 1-minute rate in standing water at 20°C. is lower for a dissolved oxygen concentration of 4.5 mg/l than for a concentration of 7.0 mg/l, but the 5-minute total count for 4.5 mg/l is considerably higher than for 7.0 mg/l. Obviously, the need for ventilation is greater at 4.5 mg/l than it is at 7.0 mg/l.

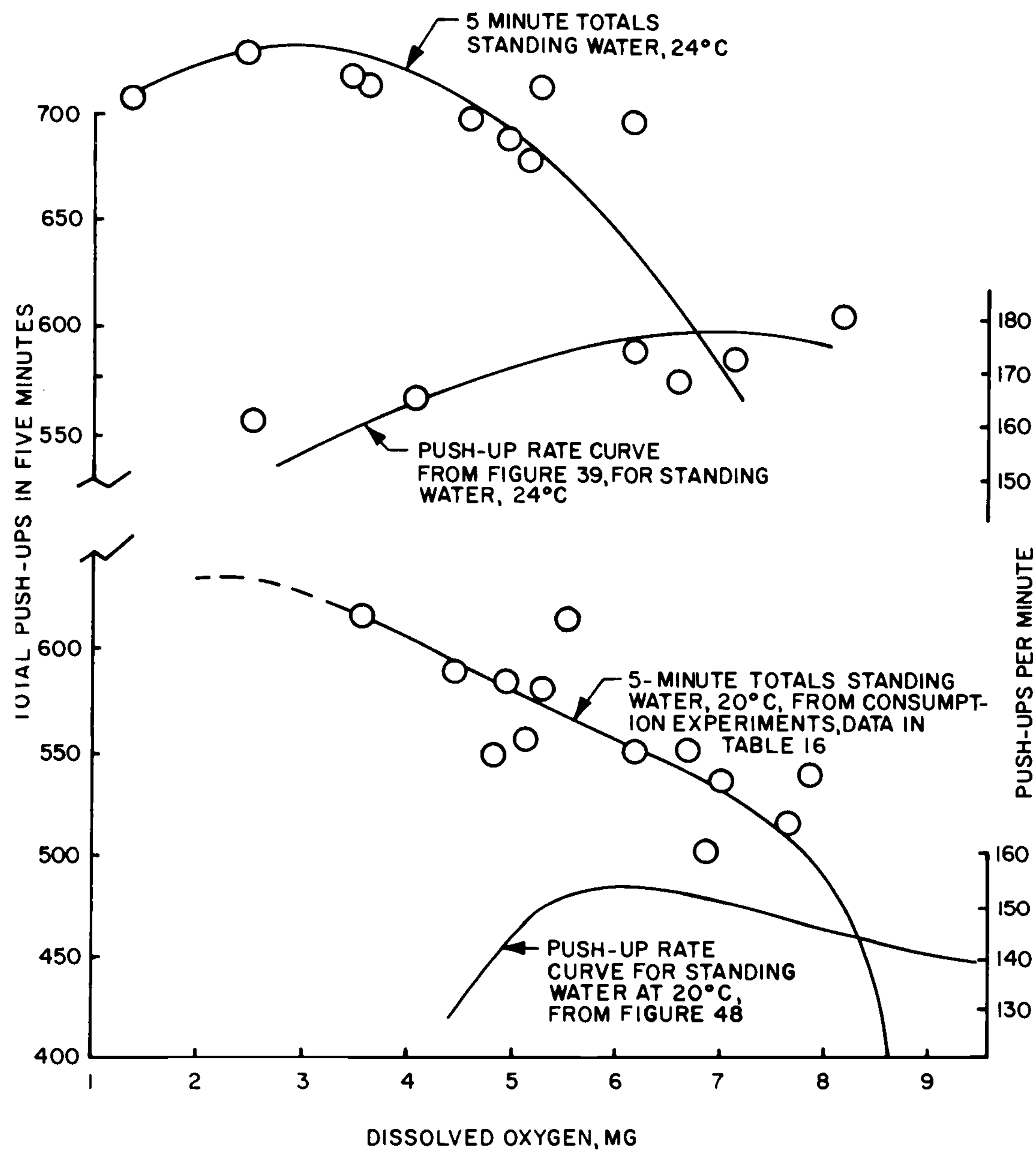

Figure 44. The relationship between 5-minute total push-up counts and 1-minute push-up rates for A. californica (Arcata) at two temperature levels. Data from experiments of 24 hours or less duration.

The higher 5-minute total counts obtained at the former concentration clearly reflect this greater need for ventilation, whereas, the reduced push-up rates do not. The curves based on 5-minute total counts at 20 and 24°C. show that the maximum counts occur at oxygen concentrations approximately equal to or less than the median tolerance limits obtained under the same conditions of temperature and velocity (see Table 14 and Figures 33 and 35). It was possible to obtain counts of push-ups at oxygen concentrations below those that are lethal within 24 hours because the naiads do not necessarily die immediately at these concentrations. At very low concentrations, however, immobilization can occur very quickly.

Acroneuria naiads may be able to adjust themselves to some low oxygen concentrations. This is suggested by a decline with time in the 5-minute total push-up counts under constant conditions of oxygen and temperature (see Figure 45). Adjustment was indicated also by the 5-minute totals observed in the artificial stream experiments in which the animals were held in screen capsules (Figure 46). McLeese (32, p. 254, 268) found definite acclimatization to reduced dissolved oxygen over a four-day period by the American lobster. However, he concluded that such acclimatization increased the survival time without having a significant effect on the lethal level of oxygen. It is known that the standard metabolic rate of fishes decreases markedly with starvation (Fry, 19, p. 37), and since this is probably true of insects also, it is possible that the decrease noted in the 5-minute total push-up counts obtained with the Acroneuria which were not fed during the experiments was a reflection of reduced metabolism due to starvation. If an increase of tolerance of low oxygen concentration actually does occur among naiads of Acroneuria, it could be at least a partial explanation for the similarity of survival percentages obtained in 24-hour experiments and longer experiments at about the same concentration of oxygen. A reduction in metabolic needs due to starvation might also be a partial explanation of this.

Measurements were made of the consumption of oxygen by naiads of A. californica (Arcata) in still water at 20°C. A summary of these and related results are given in Table 16. The oxygen consumption rate of these naiads is similar to that of naiads of a related species, Perla bipunctata, of nearly the same size, as determined by Pattee (35). He observed a consumption rate of 849 $mm.^3$ (1.21 mg.)/gram of dry weight/hr. for the latter form after the naiads were subjected to a 2-hour temperature change from 13 to 20.5°C. His experimental

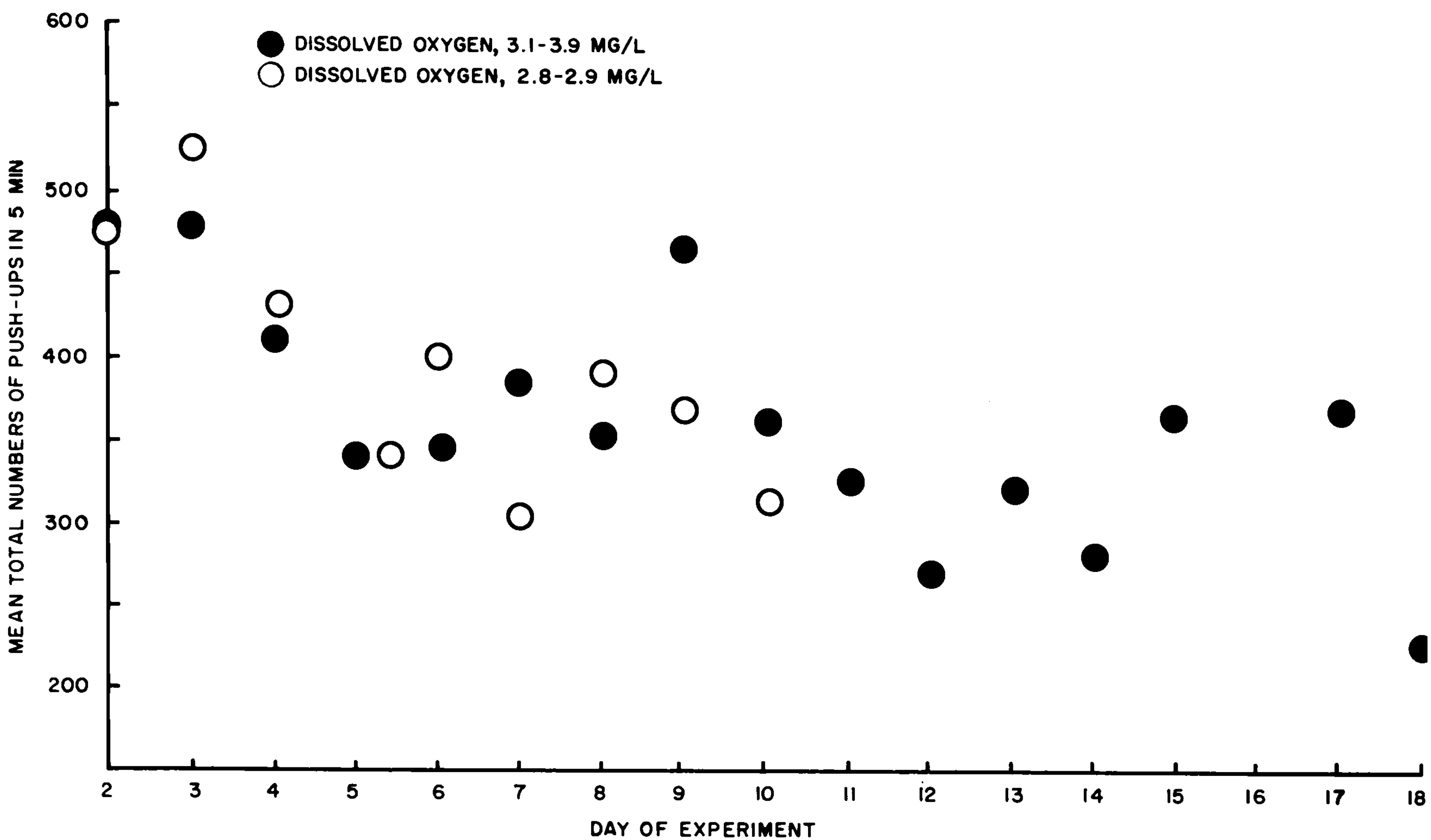

Figure 45. The decline with time in total numbers of push-ups by A. pacifica (Corvallis) in 5-minute periods in tube experiments at 20°C. and a water velocity of 0.03 fps.

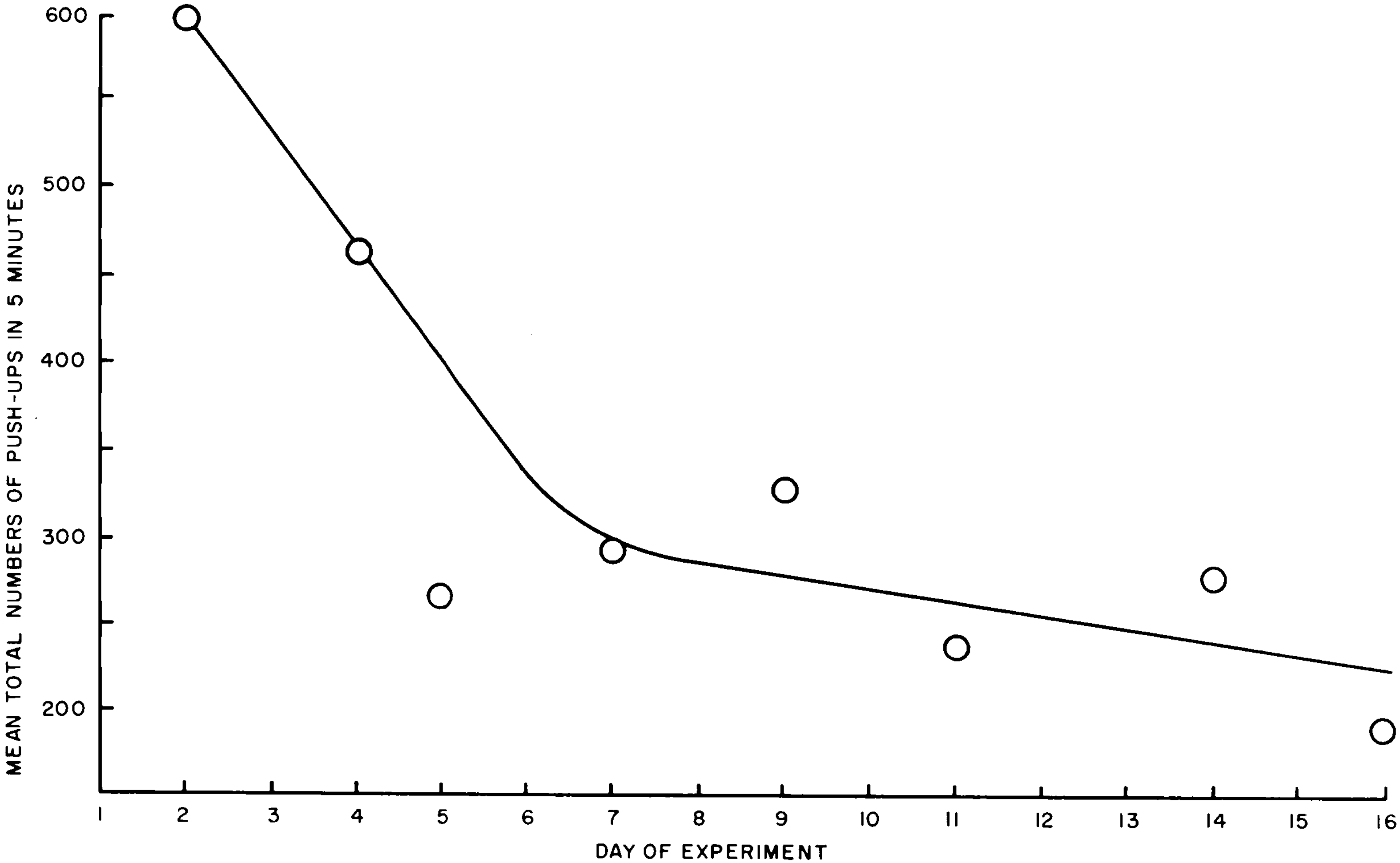

Figure 46. Adjustment with time in total number of push-ups per 5-minute period for A. pacifica (Corvallis) held in screen capsules in riffle experiment. Mean dissolved oxygen concentration, 3.78 mg/l.

method was similar to that employed in the work with <u>Acroneuria</u>. However, as he failed to give the dissolved oxygen level at which the experiments were conducted and the animals were subjected to the stated temperature change, precise comparison of his data with data on <u>Acroneuria</u> is impossible. It has often been shown (e.g., Ambuhl, 2, p. 229-252) that the consumption of oxygen among immature aquatic insects is generally higher at high oxygen levels than at lower levels. Ambuhl did describe the precise conditions of dissolved oxygen under which his determinations were made, but he did not give exact information as to the size (weight) of his test animals. As Pattee and others have shown, among individual naiads or aquatic larvae of the same species, size is a major factor in determining the rate of oxygen uptake. Also, whether the water is standing or flowing and the velocity of flowing water have much to do with the uptake of dissolved oxygen by immature aquatic insects. Because of the wide variation in experimental technique and in the animals used in the different studies which have been reported in the literature, no results were located which can be compared meaningfully with those for <u>Acroneuria californica</u>.

Figure 47 shows the relation between oxygen concentration and determined rates of oxygen consumption by naiads of *A. californica* (Arcata) in standing water at 20°C. In Figure 48, the relationships between oxygen concentration and oxygen consumption rates, push-up rates and 5-minute total counts obtained at 20°C. in standing water are compared. The consumption rates evidently were dependent, under the experimental conditions, on the oxygen concentration at all levels up to the air-saturation level, and possibly are concentration-dependent even at higher levels. For reasons to be explained presently, this suggests that the oxygen consumption rates determined may have been nearly the maximum or "active" rates for the conditions specified. Similar results indicating dependence of the oxygen consumption rates of presumably resting aquatic insects on dissolved oxygen concentration levels near air-saturation have been obtained with other organisms such as stream chironomids (Walshe, 50, p. 35-44) and, by Ambuhl (2, p. 229-241, 246-250) for several species of caddisfly larvae and mayfly naiads. Ambuhl obtained such results in experiments in which the animals were held in flowing water, and found that, for six of the seven species studied, oxygen consumption rates could be higher at high water velocities than at lower velocities, even at oxygen concentrations near air-saturation.

Table 16. Summary of Conditions and Results of 3-hour Oxygen Consumption Experiments in Standing Water at 20°C. with Naiads of *A. californica* (Arcata).

Date	Diss. Oxygen mg/l Mean	Diss. Oxygen mg/l Range	Consumption mg/g/hr[1]	No. Naiads	Naiad Mean Wet Weight mg.	Mean No. Push-ups per Minute	Mean 5-min. Total Push-up Count
4/7/60	4.82	0.85	0.52	20	214.4	136	548
2/9/60	7.86	0.74	0.97	18	125.1	153	538
2/13/60	7.65	1.16	0.99	20	171.8	154	515
2/19/60	7.00	0.81	0.95	19	131.9	151	536
2/26/60	5.57	0.85	0.78	19	161.0	156	614
3/21/60	4.44	0.50	0.48	20	126.6	136	588
5/3/60	4.92	0.96	0.72	19	192.3	134	584
5/5/60	3.58	0.65	0.59	19	167.5	146	615
5/24/60	5.14	0.83	0.62	20	183.5	145	556
6/18/60[2]	6.88	1.20	0.15	19	142.6	150	502
6/19/60[2]	5.30	0.78	0.15	13	130.2	149	578
6/20/60[2]	6.19	0.76	0.10	19	136.8	146	550
6/22/60	8.61	0.82	1.23	17	115.4	140	344
3/27/61	7.67	1.33	0.81	20	171.8	-	-
3/28/61	5.19	0.80	0.47	20	201.1	-	-
4/6/61	5.32	1.12	0.62	22	206.4	-	-
4/6/61	6.28	1.12	0.79	22	180.2	-	-
4/15/61	2.31	0.27	0.24	19	168.9	-	-
4/15/61	2.52	0.66	0.47	22	168.3	-	-

1 Milligrams of oxygen consumed per gram of dry weight per hour.

2 Many naiads emerging during these experiments; results not plotted in Figure 47.

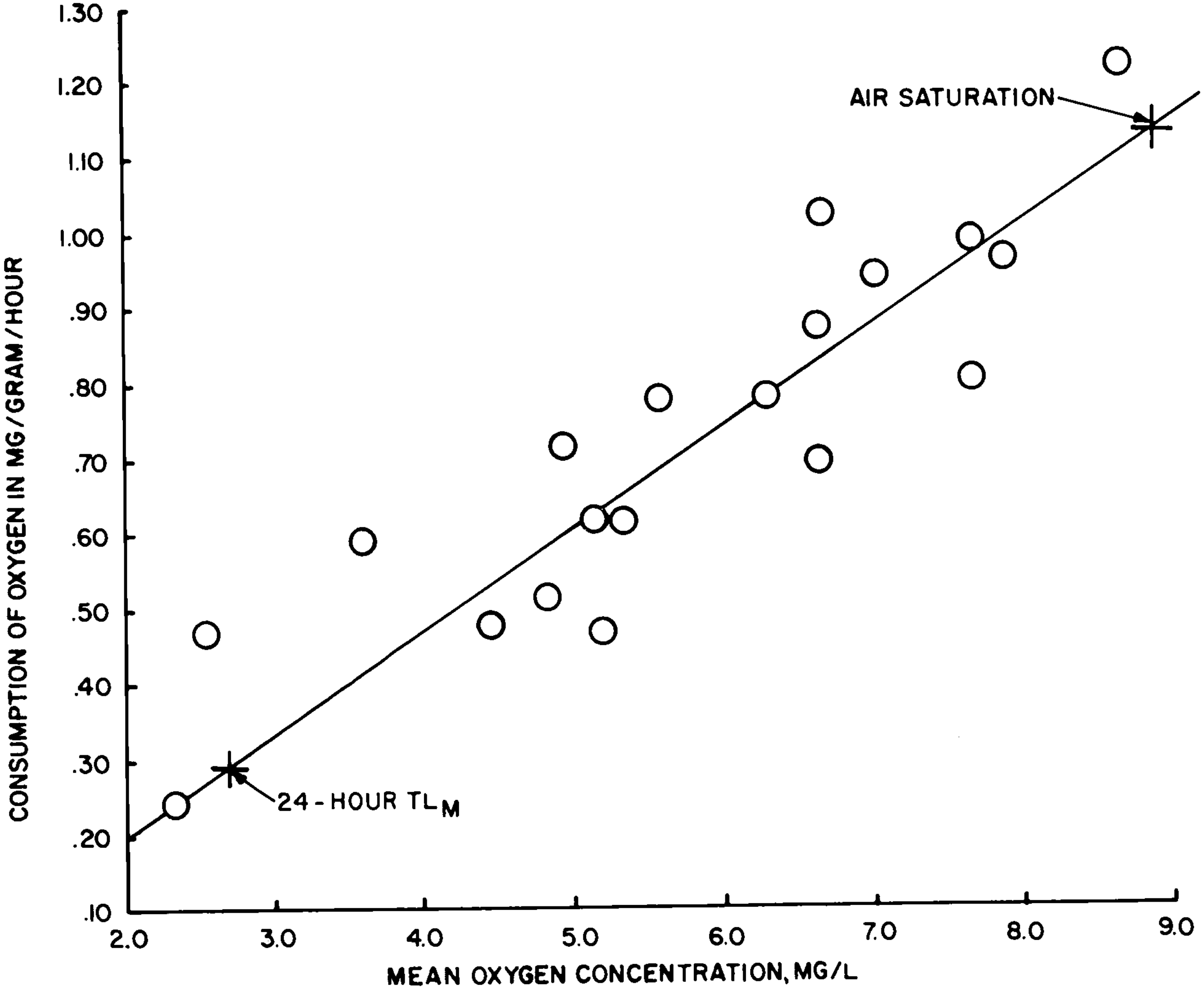

Figure 47. The consumption of oxygen by naiads of A. californica (Arcata) in standing water at 20°C.

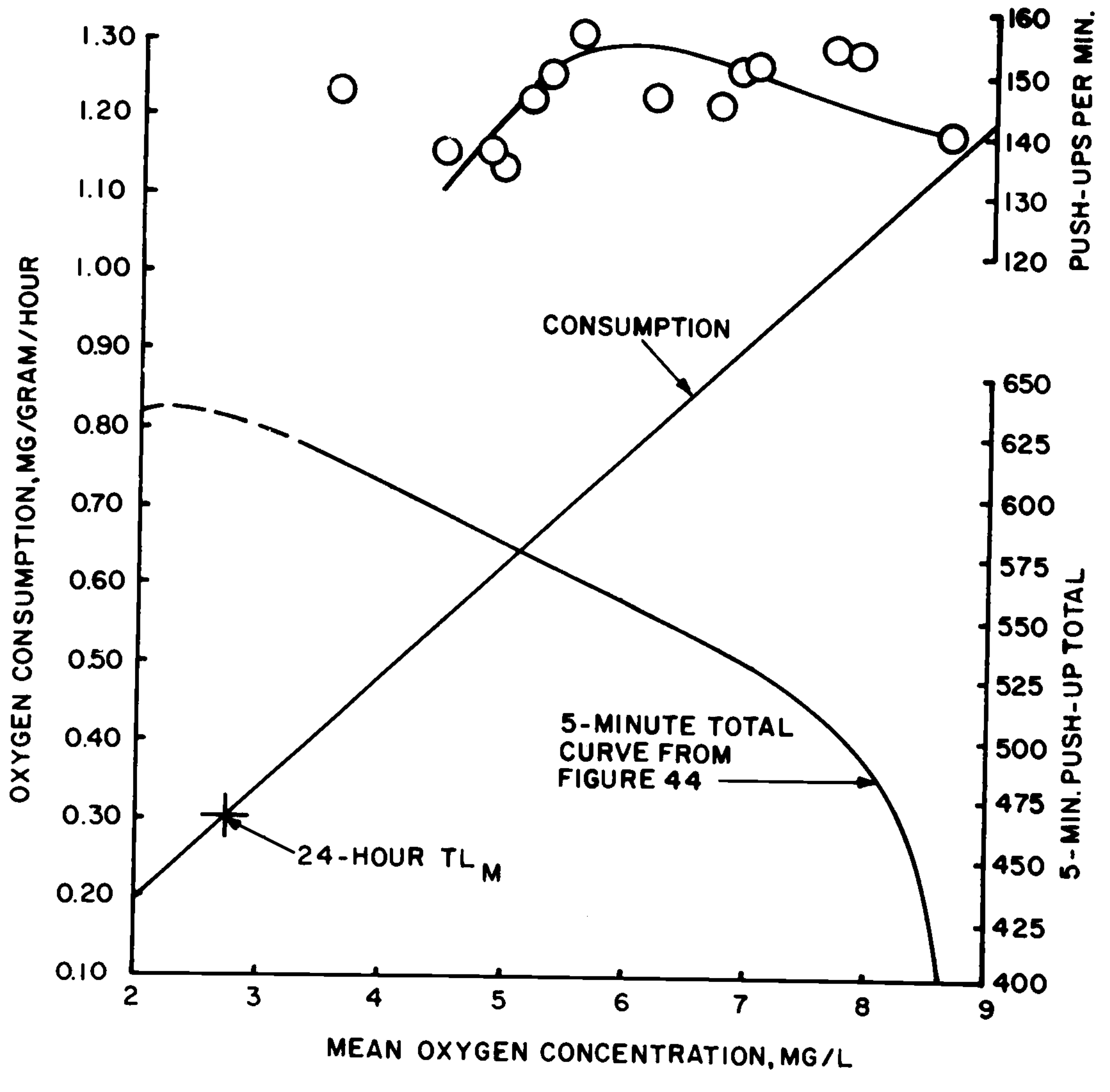

Figure 48. The relationship between curves relating oxygen consumption push-up rate. and 5-minute push-up totals of A. californica (Arcata). at 20° C. in standing water, to oxygen concentration. All determinations were made on the same groups of animals during the course of the same experiments. Data from table 16.

As Fry (18, p. 41-46) says, "respiratory dependence" (on the concentration of oxygen) among aquatic animals is generally well-expressed over a wide range of levels of oxygen only when the animals are respiring at nearly their maximum rate. According to Fry (19, p. 24) the "active rate" of oxygen uptake of fish is that rate which accompanies the highest continued level of activity under given environmental conditions. The "standard rate" of oxygen uptake of an undisturbed, unfed, and resting fish is near the minimum rate which will allow for the continued existence of the animal, and therefore, it is not dependent on oxygen concentration within the tolerable concentration range. It is not clear why the observed oxygen consumption rates of Acroneuria should be near the maximum or active rates, nor is it clear whether these rates were indeed nearly maximal. It is well-known, however, that the oxygen consumption rates of fish often remain at a high level near the maximum level for a long time after they have been handled and placed in a respirometer. It is possible that the observed oxygen uptake rates of Acroneuria also were higher than their normal "resting" rates for the specified temperature and oxygen conditions. If this was the case, the observed dependence of the uptake rates on oxygen concentration at levels near the air-saturation level may not occur among undisturbed, resting naiads in their natural environment. Another possibility that must be recognized is that in many invertebrate animals that show dependence of oxygen consumption rates on oxygen concentration over a wide concentration range, anaerobic metabolism becomes increasingly important as the oxygen supply decreases. This could make possible the continued survival of the animals at oxygen concentrations and water velocities insufficient for the maintenance of normal, resting oxygen consumption rates. However, the increasing importance of anaerobic metabolism need not be assumed for explaining respiratory dependence of seemingly resting animals until it has been conclusively demonstrated that the oxygen consumption rates being measured are not, for some reason, essentially active rates, reflecting metabolic rates far above the basal metabolic rates of the animals at the experimental temperatures. The need for further investigation of the oxygen consumption rates of aquatic insects and their dependence on oxygen concentration and water velocity is clearly indicated.

DISCUSSION

Artificial streams are believed to be useful for the study of a variety of ecological problems. One of these is the problem of identifying important ecological factors in polluted streams and determining the significance of these factors. The factors responsible not only for the mortality and consequent reduction in numbers of stream organisms in polluted waters, but also for changes in the productivity of the organisms presumably can be investigated effectively through experiments with artificial streams. The principal objective of the present work was the development of satisfactory procedures for the evaluation, through the use of artificial streams, of the long-term resistance of representative stream organisms to the pollutional conditions that may occur in streams receiving pulp mill waste liquors and for the identification of the specific causes of death of animals under these conditions.

The experiments were undertaken also in order to explore the usefulness of artificial streams in the development of acute toxicity bioassay "application factors" for the regulation of the concentrations of wastes of variable toxicity in waters receiving the wastes. As noted earlier, such use of artificial streams has been suggested by Warren and Doudoroff

(51, p. 213A-214A). An application factor is that fraction (or, as defined and used by some authors, the reciprocal of the fraction) of the median tolerance limit (TL_m) of an acutely toxic waste, as determined by standardized 24, 48, or 96-hour bioassays with selected test animals, which is deemed a permissible concentration of the waste in receiving waters. If, for example, there are sufficient reasons for believing that a concentration of 0.1 times the 48-hour TL_m of a particular kind of waste for some test animal can be maintained for a long time in a natural stream without harm to any of the valuable organisms or their production in the stream, whereas higher concentrations are likely to be injurious because of toxicity of the waste, then the fraction 0.1 (or its reciprocal, 10) might be prescribed as a suitable application factor for the 48-hour TL_m of this waste. The concentration of the waste in a receiving stream then would not be permitted to rise above one-tenth the 48-hour TL_m, determined before discharge of the waste. So long as this concentration level (48-hr. TL_m x 0.1) is not exceeded, it would be assumed that organisms of value are being adequately protected from toxic effects of the waste, unless or until some evidence to the contrary comes to light. Warren and Doudoroff (51, p. 213A-216A) discuss in detail the use of application factors, their determination, and their verification in connection with the disposal of pulp mill wastes.

The objectives of the artificial stream experiments cannot be said to have been fully achieved. Failure to arrive at many definite conclusions is ascribable partly to some inherent limitations of the experimental approach in question, but is believed to be attributable largely to certain defects of the exploratory experiments that can be corrected or avoided in similar future investigations.

The experimental results presented in this thesis show that environmental changes which are known to take place in natural streams receiving sulfite and kraft pulp mill wastes can be reproduced in artificial streams, and that these streams can be useful in evaluating the resistance of some life stages of some aquatic organisms to the adverse conditions produced by these wastes. It has also been shown that a set of artificial streams can be a valuable aid to the identification of various factors responsible for losses of animals in waters receiving pulp mill wastes and estimation of the relative importance of these factors, even though these objectives may not be fully attainable through artificial stream experiments alone.

The fractions of the 24-hour TL_m's of kraft waste that were maintained in the artificial stream experiments reported were, in a sense, trial application factors for the 24-hour TL_m. No significant increase in the mortality rate of Acroneuria naiads was demonstrated at waste concentrations less than 0.1 times the 24-hour TL_m for the guppy; whereas tested concentrations higher than this were clearly detrimental to the insects, the toxicity of the wastes being apparently a dominant cause of increased mortality. However, since toxicity bioassay application factors must be designed so that their use would prevent damage to the productivity of all valuable aquatic organisms, experiments such as those described herein are not deemed alone sufficient for the development of such factors, and none can be prescribed yet for kraft waste.

While the results of the present work fall far short of full realization of its stated objectives, it is apparent, in the light of the experience gained, that improvements in the experimental apparatus used and in the design and conduct of the experiments would have made it possible to obtain more valuable results. The main topic of this discussion will be the design of apparatus and experiments for similar studies that might be undertaken in the future with the same objectives in mind. The design and use of artificial streams for productivity and reproduction studies also will be considered. First, however, the reasons for using artificial streams, that is, the specific advantages to be gained by their use, will be reviewed, and the limitations of this approach to ecological problems also will be discussed.

Natural stream environments usually are so complex and variable that understanding or interpretation of observations made on them can be extremely difficult or impossible without reliance on simplified models. Environments in some laboratory apparatus may be so simple that the results of experiments with such apparatus may be difficult to relate to natural phenomena, and so may not be very meaningful, ecologically. Experiments with artificial streams combine some of the major advantages of both field studies on natural streams and laboratory studies with simpler apparatus, and although all the disadvantages of the two last-mentioned kinds of studies obviously cannot be avoided, the artificial stream studies can provide information not readily obtainable by other means.

One of the major advantages of artificial stream experiments over field studies on natural streams is that conditions in artificial streams, such as the concentration of introduced wastes, concentration of dissolved gases, water velocity, temperature, illumination, and community composition, can be controlled or modified at will as they cannot be controlled or modified except at great expense in natural streams even of correspondingly small size. The recirculation of water in artificial streams reduces the cost of water treatment or modification. Another advantage is that a series of artificial streams that are identical in every respect except for one or more controlled variables can be readily established and studied simultaneously to determine the effects of these factors. For example, the effects of different waste concentrations in a graded series can be compared. Sampling, observation, and reliable enumeration of organisms and determination of the fate (death, emergence, etc.) of the organisms in artificial streams is comparatively easy. The interpretation of observations on artificial streams is, of course, greatly facilitated by the relative simplicity of the environments and of communities in these streams, as compared with natural streams and their highly complex communities.

Environmental conditions in artificial streams with natural bottom materials and some of the other features of natural streams are more likely to be suitable for prolonged survival of many stream organisms than are those in simpler laboratory apparatus lacking any even superficial resemblance to natural streams and offering little or no opportunity for the organisms to select suitable niches. Also, the behavior and metabolism of many animals can be expected to be more nearly normal when the animals are held in artificial streams than when simpler laboratory apparatus is used, and consequently, the results of tests with such streams may be deemed more applicable to natural streams than are results obtained with the simpler apparatus.

Some indirect effects on organisms of changes in water quality can be expected to occur in artificial as well as in natural streams and can be studied effectively in the artificial streams, but cannot be reproduced in overly simplified and unnatural environments. Valuable studies of the degree of "slime" development in different concentrations of pulp mill waste at known and constant water velocities and temperatures can be and have been carried out using simple tubes with flowing water and suitable attachment surfaces or subtrates. However, the development of realistic "secondary bottoms" composed mainly of slime can be observed and their effects on benthic animals can be studied only in artificial streams with at least some natural bottom materials, or in natural streams. Meaningful studies of the effects on the survival or production of stream animals of the elimination or increased abundance, in the presence of added wastes, of important food organisms or competitors can be carried out using artificial streams, but not with much simpler apparatus.

It is realized, however, that the relative simplicity even of artificial stream environments and communities, as compared with natural stream environments and communities, can render the artificial streams unsuitable for the indefinite survival of many stream organisms, or for the completion of their life cycles. Furthermore, when artificial streams in which only very simple communities have been established are used for the investigation of the effects of pollutional materials on stream productivity, there is danger that the results will be misleading. A toxic or other pollutional material may eliminate or seriously reduce the production of the only food organism of key animals in the community, thus drastically and excessively reducing the production of animals that depend on it for their food supply either directly or indirectly. On the other hand, in a more complex natural stream community, when one important food organism is eliminated by a pollutant, its place may be taken by another species that is more tolerant of, or even favored by, the pollutional conditions, but equally suitable as a food organism, so that the production of the animals dependent on these organisms for food may not be adversely effected and may even increase. Thus, the application of the results of artificial stream studies to problems in natural streams may not always be possible. The danger in making such application is reduced as the structural complexity of the artificial streams and communities is increased. However, highly complex artificial streams and communities could be difficult to study, and their complexity would tend to defeat the main general purpose of such streams.

Studies with laboratory apparatus even simpler than the artificial streams used in the present investigation may, of course, be required for some purposes. Though much simpler than natural stream environments, the artificial stream environments nevertheless have complexity great enough to obscure some effects of environmental factors on organisms and render the detection and precise evaluation of these effects difficult. For example, the influence of water velocity on the dissolved oxygen requirements of *Acroneuria*, which was successfully studied by placing the animals in tubes or jars, could not be effectively evaluated through artificial stream experiments alone, because the velocities to which the animals might be exposed in the streams were too uncertain and variable. Also, the problem of the degree of interaction of reduced dissolved oxygen and the toxicity of pulp mill wastes was not solved through the artificial stream experiments, and doubtless more reliable evidence of such interaction and measures of its magnitude could have been obtained through flow-tube or jar experiments with precisely and independently controlled oxygen and waste concentrations as the only variables of consequence.

The results of the artificial stream experiments pointed up the need for precise information on the tolerance of the test organisms to reduced dissolved oxygen. _Acroneuria_ naiads were observed dead and dying in the artificial streams when oxygen concentrations were low, and it was apparent that reduced oxygen concentration could be an important factor or cause of this mortality, although some of the mortality evidently was due wholly or partly to other factors, such as toxicity of the wastes. Unlike the artificial stream experiments, the tube and jar experiments were designed to provide and did provide precise information on the low oxygen tolerance of _Acroneuria_ naiads (the principal test animals in the stream experiments) as related to temperature and water velocity. Although helpful, the information obtained in the tube and jar experiments could not be fully employed in interpreting the results of the stream experiments because of inadequacy of the information gathered on naiad movement and location in the streams, and water velocity and dissolved oxygen at each location. The results of studies on streams, natural or artificial, can probably not often be thoroughly understood in the absence of precise information on the specific water quality requirements of the organisms involved. Conversely, information on the specific requirements of these organisms may not be of much use in interpreting the results of stream studies if precise and relatively complete information on the streams is not available. Clearly, there is need for a variety of approaches to problems of stream ecology.

A number of improvements of apparatus and of experimental design or procedure might have increased the value of the results obtained or facilitated the conduct of the experiments with artificial streams. For example, higher water velocities might have been maintained in some tests (by using pumps of greater capacity, or by reducing the depth of the water in the troughs) in order to explore the influence of variations of water velocity and also the possibility that a greater variety of typical riffle-dwelling organisms can be held and tested at higher velocities. As Ambuhl (2, p. 133-264; 3, p. 390-395) and Zimmermann (58, p. 1-81) have shown, the importance of water velocity in stream ecology can hardly be overestimated. In the present study, artificial streams were not used for exploring fully the extent to which differences of water velocity may influence the survival of organisms in the presence of pulp mill wastes. The "pool" and "riffle" experiments reported did not consistently yield markedly different results; but the detection of possible important effects of the water velocity differences was complicated by uncontrolled variables such as water temperature.

A surface velocity of 0.4 to 0.5 f.p.s. was maintained in the artificial stream riffles. Higher velocities were attainable, but only by reducing the depth of the water in the troughs. The velocity which was maintained was considerably lower than the 1 to 3 f.p.s. velocities characteristic of riffles in many natural streams receiving pulp mill wastes. Many important aquatic animals normally inhabit riffles flowing at these or higher velocities. Wu (56, p. 596) reported that Simulium larvae were found in natural habitats at surface velocities of 0.56 to 2.75 f.p.s. Scott (41, p. 351) reported that the modal surface velocity in riffles selected by some caddisfly larvae such as Rhyacophila dorsalis and Hydropsyche fulvipes in a natural stream was above 40 cms./sec. (about 1.3 f.p.s.) and that a large part of the population of R. dorsalis in this stream occurred at surface velocities over 100 cms./sec. (about 3.3 f.p.s.). It is obvious that much higher velocities than those used in the present study will need to be produced in artificial streams for some future studies of the importance of velocity in polluted waters.

The riffle currents in the experiments reported herein were produced by electric pumps; but while pumps of sufficient capacity are capable of producing fast currents, they have the disadvantage of raising the temperature of the water by as much as several degrees and of being destructive to some small organisms or materials that cannot be prevented by screening from passing through them. In order to avoid undesirable heating of the water and damage to organisms, currents may be produced by other devices such as paddlewheels, using two parallel troughs, connected at both ends, as artificial streams. Paddlewheels can be constructed so as to make unnecessary the screening of the water being recirculated by them for the protection of most organisms; but water velocities attainable by such devices probably are limited, especially when the flow of the water is obstructed by rubble on the artificial stream bottoms. The installation of screens in the troughs and the cleaning and maintenance of them in the presence of heavy growths of slime in the present experiments presented difficulties. Garrison (20, p. 5) used variable speed paddlewheels in some artificial stream studies of the metabolism of algal communities; but with them, the maximum water velocity attainable without excessive splashing was only about 1 f.p.s. Motor driven propellors of the general type used by Ambuhl (2, p. 244) for physiological studies in flow pipes, or perhaps Archimidean screws, might be more suitable for use in some artificial stream apparatus.

Water temperatures varied widely between the artificial stream experiments, and this no doubt accounted for much of the variability of the results. The lack of means for adequate control of temperature precluded satisfactory assessment of the importance of temperature as an environmental factor in these experiments. The heating or cooling of large quantities of fresh water continually introduced into the streams can be very costly, but the cost of temperature control can be greatly reduced by the use of counterflow heat exchangers, whereby the effluents from the streams are used for heating or cooling the fresh incoming water. Even if water temperatures can be controlled, it may not be desirable to perform experiments at the same temperature during different seasons of the year, because the responses of many organisms at any given temperature may vary with the season. For example, Edwards (15, p. 53-64) states that the oxygen consumption rates of arthropods determined at the same temperature are often higher in winter than in summer. For studies of the effects of natural variations in temperature, it may be necessary deliberately to simulate such variations in the artificial streams.

In the artificial stream experiments, the river water that entered the troughs was not screened nor filtered, and numerous small organisms were introduced into the streams with this water. In these experiments, such introduction of small organisms was not deemed undesirable and probably it is even advantageous in most experiments on the tolerance of organisms to pollutional conditions. Small drifting organisms may serve as food for larger test animals in artificial streams, and polluted sections of natural streams no doubt normally receive large numbers of drifting organisms from upstream sections or tributaries. Filtration of inflowing water would be undesirable when some of the test animals are forms that depend on materials brought in with the water supply for their nourishment. On the other hand, the inadvertent introduction of animals of the same species as those that have been deliberately introduced into the artificial streams and counted, or of carnivorous forms that might feed on the deliberately introduced smaller organisms, thus increasing their mortality, clearly is also undesirable. The complete elimination of all living organisms from the water entering a trough may be difficult, as even sand-filtered water may contain algal cells and animal sex products, but screens of suitable mesh size usually should be adequate for removal of any undesirable larger organisms.

The amount of food obtained by organisms may influence their tolerance of some adverse conditions. Suitable food organisms might have been introduced into the artificial streams regularly to ensure adequate and fairly uniform availability of food for the test animals such as *Acroneuria* and thus perhaps reduce cannibalism. Had unrestricted food supplies in all streams been thus ensured, observations on the growth rates of insects at different tolerable waste concentrations might have been feasible and profitable.

Precautions should be taken to avoid the unnoticed loss of animals from artificial streams by crawling out or emergence. In the early "pool" experiments of the present study, the troughs were covered with panels of glass. Since there was no indication of any attempt by immature organisms to crawl out of the troughs during these experiments, and since the panels made viewing difficult and were not easy to handle, these covers were not used and apparently were not needed to prevent crawling out in the later experiments. Continual illumination may have been the main deterrent to the crawling out of stonefly naiads from the troughs. However, it may not have this effect on other species, and furthermore, normal diurnal periodicity of illumination of artificial streams is now deemed usually preferable. To ensure against loss of any aquatic insects, screens or cages probably should usually be placed over the troughs. Even when screening appears to be adequate, the area around an artificial stream should be carefully examined periodically so that animal escapement does not go unnoticed.

It would have been advantageous to have been able to observe organisms and their environment under the rocks in the artificial streams, particularly at times when "slime" was being produced and when the test animals were in distress due to consequent unfavorable conditions in the streams. Observations on the vertical distribution of slime produced with different concentrations of waste liquors and on the location and behavior of test animals under the rocks and slime under the conditions of reduced dissolved oxygen would probably have been particularly instructive. Since push-up rates of _Acroneuria_ are dependent on water temperature, water velocity, and dissolved oxygen, measurements of temperature and oxygen along with observations on push-up rates might have provided a means of indirectly determining the water velocities to which individual naiads were exposed. Coverable transparent panels in the walls of the troughs would have made some of the desirable observations possible.

It is now realized that the data collected in the course of the artificial stream experiments were not entirely adequate. Accurate data on the movement of water among bottom materials, before and during the introduction of waste into the streams, might have been helpful in determining the relative importance of low dissolved oxygen as a cause of mortality of <u>Acroneuria</u> naiads, since water velocity was found to be an important factor influencing the low oxygen tolerance of the naiads. The accurate measurement of low velocities of water flowing among bottom materials is not easy, but the photographic method used by Ambuhl (2, p. 141, 142) may be suitable for this purpose if the stream channels used are wholly or partly constructed of glass. In this method, acetyl cellulose particles suspended in the water are illuminated and photographed for the determination of their velocity.

Additional dissolved oxygen analyses of water samples obtained as nearly as possible from the immediate vicinity of the test animals in the artificial streams also would have been helpful. More attention could well have been given to the vertical distribution of oxygen at various stages of each experiment. Perhaps sampling at fixed points would have provided data that would have shown more clearly the changes in the oxygen concentration at various points in the troughs with time. A method for dissolved oxygen determination more reliable in the presence of pulp mill wastes than the one used, perhaps the "iodine-difference" method of Ohle (34), should be used in future studies of pollution by these wastes.

Some information was obtained on the toxicity of kraft waste by artificially adjusting upward in one experiment the concentration of dissolved oxygen. More such experiments should have been conducted to more firmly establish that, in the artificial streams, kraft waste in tested concentrations can cause death of aquatic insects even when the concentration of dissolved oxygen is high. Such additional tests perhaps would have shown definitely whether interaction between reduced dissolved oxygen and other effects of the kraft waste and sulfite waste contributed to the mortality that occurred among the aquatic insects in the presence of these wastes. Alderdice and Brett (1, p. 792) found that lowered dissolved oxygen increased the toxicity of kraft mill effluent to young salmon in sea water, but it is not known whether such interaction occurs in aquatic insects.

The results of acute toxicity bioassays of kraft waste provided the basis for the selection or standardization of the concentrations of this waste to be used in the long-term stream experiments. Partly because of difficulties encountered in conducting some preliminary bioassays with stonefly naiads and because published standard methods for bioassays with fish, but not aquatic insects, were available for use, the standardization of kraft waste concentrations maintained in the experiments, in which the principal test animals were aquatic insects, was based on the results of bioassays with fish. It is realized that the waste components toxic to fish may not be the ones toxic to insects, or at least the degree of their toxicity may be different for the different animals. Therefore, uniformity or standardization of the tested waste dilutions, with respect to their toxicity to insects, might have been better achieved by using insects as test animals in the acute toxicity bioassays of all kraft waste samples. However, trial application factors for use in a series of artificial stream experiments must be based on bioassays with some single species if the results of the experiments are to be strictly comparable. This species must be one that can be conveniently used and is likely to be used routinely in testing wastes for waste disposal control purposes. For the regulation of waste discharges to natural streams, a suitable fish will likely be used ordinarily for assaying the acute toxicity of a waste, even though the application factor for applying the results of the bioassay is intended to provide for the protection not only of fish but also of other valuable aquatic organisms. For this reason, the use of a fish such as the guppy as the test animal for standardization of kraft waste dilutions in the artificial streams is deemed quite appropriate and justifiable.

Artificial streams are believed to be useful not only in studies such as those reported herein, but in other kinds of studies as well. Studies of the trophic dynamics of stream communities and of the manner in which energy flow patterns and plant and animal production rates are influenced by environmental changes can be carried out using artificial streams. The effects of water pollutants or other environmental factors on the success of reproduction of representative stream organisms also can be studied in artificial streams.

Artificial stream experiments can be profitably undertaken to determine the effects of pollutional materials or other factors on growth rates or production of animals not only in the presence of unlimited food supplies for these animals but also in the presence only of limited, naturally produced (self-propagating) food supplies whose abundance in the streams may be influenced by the added wastes or other environmental factors. Thus, the indirect effects on animal production of pollutional conditions that influence the production of food organisms in a stream, as well as the more direct effects on the growth of the animals of conditions that influence their appetite and ability to assimilate food, can be explored and evaluated.

It probably matters little that some species of fish-food organisms are reduced in numbers or eliminated by a pollutant as long as the production of valuable fish remains undiminished. The reduced numbers of species and reduced quantities of algae observed in the artificial streams receiving sulfite and kraft wastes, as compared with those observed in the control streams, indicate that these wastes can reduce the production of these organisms. Whether the reduced abundance of algae would result in reduced production of carnivorous fishes in a stream is one important question not answered by the present investigation. As has already been stated, in some experiments the presence of sulfite waste resulted in increased numbers of chironomids and of a cladoceran, perhaps partly because this waste promoted great abundance of bacteria and other heterotrophic microorganisms that could be utilized as food by these animals. However, the increased production of these heterotrophs and of the larger fish-food organisms that feed on them may take place in streams at the expense of herbivores possibly more valuable as fish food than those that are favored by the presence of the waste.

When the influence of pollutional materials on animal production or reproduction is to be investigated, the artificial stream method will provide for such studies the same advantages that it has for studies of long-term tolerance to pollutional conditions. However, certain precautions, perhaps not so important in some other stream studies, will need to be observed in productivity and reproduction studies. Since the kinds and amounts of food available are key factors in the production of organisms, in studies of the effects of pollutional materials on the utilization by organisms of food available in unlimited amounts and in suitable forms, special provision for the maintenance of such food supplies may be necessary. Even when unfiltered and unscreened river water being supplied to artificial streams provides much of the food necessary for organisms in the streams, the periodic addition to the streams of foods selected on the basis of known food habits of the animals tested may be necessary to ensure unrestricted diets.

When animal production is to be dependent on the food produced in an artificial stream, care obviously must be taken to prevent the introduction of any additional food via the river water supply. Filtration or proper screening of the water supply is then essential. In streams with circulating water, destruction of organisms whose production is to be measured and of their foods will need to be prevented by the use of paddlewheels or other harmless water-circulating devices, or by proper screening of pumps or propellors used for circulating the water. Since plant production on which animal production may depend is largely dependent on the available supply of energy in the form of light, some control over illumination will usually be desirable, and exact measurements of illumination may be necessary.

When there is any considerable mortality of test animals in the streams during experiments on the growth rates and production of these animals, all the animals of one kind must be nearly identical with respect to initial size, or individual animals must be suitably marked for ready recognition and weighed separately at the beginning and end of a test.

Studies of the effects of pollutants on the production of plants and animals in streams having complex, natural communities, under conditions varying seasonally in a natural way, may need to be conducted in completely natural streams or in natural streams some of whose features are controlled, but such studies involve many difficulties.

Studies of the influence of pollutional conditions on the reproduction of some aquatic animals can and probably should be carried out in artificial streams; but no special attempt was made to study this problem in the present investigation. Some normal stream bank and bottom vegetation would probably have to be provided in the stream environment for these studies, since these features may be essential for the successful development, mating, and egg-laying of some aquatic insects. Some dragonflies and some hemipterans insert eggs into the living tissues of aquatic plants. Some caddisflies and some neuropterans deposit eggs on branches overhanging the water surface. The sub-adults or adults of many species normally use streamside vegetation for resting or developmental purposes. Since the reproductive phases of the life histories of many aquatic forms are completed out of the water, large screened enclosures over artificial streams will probably be necessary for adequate control and surveillance of these forms during these phases.

For more or less complete ecological studies of the effects of added waste materials or other environmental factors on streams and on a wide variety of stream organisms, more than one approach to the problem clearly may be desirable or necessary. Approaches to the problem other than those taken in the present study, and possible improvements in the methods and materials used in this study, have been suggested in the course of the foregoing discussion. The use of artificial streams for studies of the ecology of unpolluted streams, or for studies of streams receiving pollutional materials, appears to be feasible and may be often desirable or necessary. Much is yet to be learned about the use of such streams for various research purposes, and the further investigation of their utility and development of reliable procedures for their use could be rewarding as well as interesting.

SUMMARY

Artificial streams established in wooden troughs 10 feet long and 10 inches wide were utilized for the study of the effects of pulp mill wastes on stream ecology and for the study of the long-term tolerance of representative stream organisms to these wastes and to lowered dissolved oxygen in unpolluted water. "Pool" habitats were created in some troughs and "riffle" habitats in others. Both riffles and pools were provided with stones from natural streams. Other stream materials such as detritus and suspended solids entered the troughs with the river water continually supplied to them and contributed to the make-up of the stream environments. The river water was supplied to the troughs at the rate of about 1500 ml. per minute in most experiments. In the riffle troughs, surface currents of about one-half foot per second were produced by electric pumps that continually recirculated the water in these troughs.

Crayfish, snails, aquatic insects, and fishes in known numbers were placed in the streams. Some species of plants and animals, introduced with the river water entering the troughs, became naturally established in the streams. Naiads of the stoneflies _Acroneuria pacifica_ and _Acroneuria californica_ were the principal test animals.

Sulfite process or kraft process pulp mill waste liquor was continually added at constant rates to the streams in most experiments. Constant concentrations of sulfite waste liquor solids in the streams were maintained by standardization of the liquor on the basis of its total solids content. Sulfite waste concentrations tested were from 300 to 1,200 mg/l standard waste liquor of 10 per cent total solids content. Kraft waste liquor, prepared from combined condensates and black liquor, was standardized on the basis of its acute toxicity to fish, mainly the guppy, *Lebistes reticulatus*, determined by bioassay. The kraft waste concentration maintained in any given trough was some fixed fraction of the 24-hour median tolerance limit of the waste, which was determined for each fresh sample. The concentrations tested ranged from 0.01 to 0.3 times the 24-hour median tolerance limit for guppies or sculpins. In some experiments no waste was added to the streams, and in some cases the concentration of dissolved oxygen in streams with or without waste was artificially adjusted by bubbling oxygen or nitrogen through the incoming river water before it entered the streams.

Most experiments were either about two weeks or one month in duration. Observations were made on physical, chemical, and biological conditions in the streams throughout each experiment. The behavior and mortality of test animals were observed during the experiments and the numbers of survivors at the end of each experiment were ascertained.

The waste materials introduced into the artificial streams often produced striking changes. "Slime," composed largely of Sphaerotilus natans, in some cases markedly altered the stream habitat by complete or nearly complete blanketing of bottom materials. The development of slime progressed gradually from the beginning of the introduction of pulp mill liquor to a "climax" condition in about 10 or 12 days. The final degree of development was different at different concentrations of waste liquor, but slime was produced at all concentrations of sulfite waste that were tested. None or very little was produced at a kraft waste concentration of 0.01 times the 24-hour TL_m for the guppy, but it was produced at all higher concentrations tested. Generally, higher concentrations of either waste produced greater amounts of slime. When a distinct "secondary bottom" of slime was produced, the flow of water among the bottom materials was reduced. The reduced flows and the decomposition of organic materials resulted in greatly lowered oxygen concentrations below the blanket of slime. Lowered concentrations under the slime often forced stonefly naiads and other organisms into the open on top of the slime, where there was more oxygen and a definite current. *S. natans* grew abundantly on some insects, even at times when the concentration of pulp mill waste was low and when the general development of slime on bottom materials was slight.

The accumulation of detritus and inorganic matter was considerably increased by the filtering action of slime growths in the streams receiving pulp mill wastes. In some of the sulfite waste experiments, chironomid (tendipedid) larvae became abundant, more so in the streams receiving waste than in the controls. The accumulation of material and the abundance of bacterial slime, by providing a suitable substrate and nourishment, may have been factors in the successful establishment of these larvae. The cladoceran, *Eurycercus lamellatus*, became very numerous in "pool" environments in one set of sulfite waste experiments; the number being greater the higher the concentration of waste.

Perhaps because of the greater toxicity of kraft waste, neither chironomids nor cladocerans were ever abundant in streams which received this waste. Both sulfite and kraft wastes greatly inhibited the development of growths of algae of most or all kinds that were present in the control streams. Some loss of steelhead trout and king salmon occurred in experiments with both sulfite and kraft waste, even though the concentrations of dissolved oxygen never were reduced below the tolerable level. In experiments with sulfite waste the growth rates of steelhead trout were less than they were in control streams even when no fish died.

In general, lower dissolved oxygen concentrations were associated with higher concentrations of kraft waste in the streams that received this waste, but stonefly naiads showed distress or died in streams receiving high concentrations of waste before the concentrations of dissolved oxygen were reduced to a lethal level. In other experiments, at corresponding dilutions of the waste, naiads died in the streams even though the oxygen concentrations were held well above the lethal level by oxygenation of the inflowing water. In sulfite waste experiments in which high oxygen concentrations persisted or were maintained by oxygenation of the water, markedly increased mortality of insects was not observed even at relatively high waste concentrations. However, a comparison of survival-oxygen concentration curves for naiads of <u>Acroneuria</u> and <u>Pteronarcys</u> and for limnephilid larvae, in streams receiving sulfite waste, with corresponding curves obtained with unpolluted river water in which dissolved oxygen was reduced by means of nitrogen, indicates that low dissolved oxygen was not alone responsible for the mortality that occurred in the presence of sulfite waste. Lower oxygen concentrations were generally associated with the higher sulfite waste concentrations in the experiments, but the mortality of the test animals in the presence of the waste was distinctly greater than that observed at the same reduced levels of dissolved oxygen when no waste was present. A comparison of the survival-oxygen concentration curves for Acroneuria and Pteronarcys for kraft waste and sulfite waste experiments suggests that at the same reduced oxygen levels there may have been higher mortality of the animals in streams receiving kraft waste than in those receiving sulfite waste, but because of differential chemical interference by the two kinds of waste with oxygen determinations, the evidence is not conclusive.

No marked difference in susceptibility to the lethal effects of the two wastes at the same oxygen concentrations in the streams was observed between Acroneuria naiads, Pteronarcys naiads, and limnephilid larvae. However, in one experiment with kraft waste in which the incoming river water was oxygenated and in which the dissolved oxygen remained at a fairly high level, unexpectedly high survival of Pteronarcys naiads was observed. The death rates of Acroneuria were usually slightly higher than those of the two other insects, but the difference is attributed largely to cannibalism and predation from which the other two forms did not suffer.

It has been concluded that increased mortality of insects in the presence of sulfite waste was due largely to reduced dissolved oxygen, but probably was due in part also to some other survival-depressing factor or factors such as toxicity of the waste interacting with reduced dissolved oxygen, or slime growths on the naiads, which may interfere with their respiration. The increased mortality in the kraft waste tests which was observed only at concentrations above 0.1 times the 24-hour TL_m for the guppy apparently was due largely to the toxicity of the waste, but other factors such as reduced dissolved oxygen may well have been partly responsible also.

Because lowered dissolved oxygen was observed to be such a salient feature of the artificial streams receiving pulp mill wastes, and because inadequate oxygen was shown to be a major cause of mortality or an important factor contributing to the mortality of animals in these streams, a series of experiments on the oxygen requirements and ventilatory movements of naiads of <u>Acroneuria</u> were conducted. The low oxygen tolerance of <u>Acroneuria californica</u> naiads from the Arcata, California area, at temperatures ranging from 2.2 to 24°C. and at water velocities from zero to 0.13 f.p.s., was determined. The 24-hour median tolerance limit (TL_m) of dissolved oxygen was estimated by the method of Litchfield and Wilcoxon (31, p. 99-113) for each tested combination of water temperature and velocity. The tolerance experiments were conducted under constant conditions either in an apparatus consisting of a glass pipe testing chamber through which water of regulated dissolved oxygen concentration and water temperature was recirculated so as to maintain desired water velocities, or in jars of standing water. The test animals were held in stainless steel screen capsules inserted into the testing chambers or jars.

In the tube and jar experiments, no uncontrolled or unmeasured variables of general importance, except perhaps naiad age, were known to exist. The prevailing water velocity and temperature, within the extremes tested, determined whether the average naiad would survive at any given concentration of dissolved oxygen. Tolerance of low oxygen concentrations generally decreased with increasing temperature and increased with increasing water velocity, other conditions being constant. The concentration of oxygen which was just adequate to keep the average naiad of _Acroneuria californica_ from the Arcata, California area alive for 24 hours in standing water at a temperature of 24°C. in a glass jar was about 3.7 mg/l. This was the highest TL_m value determined. The lowest concentration which allowed the average naiad to remain alive for the same length of time in water flowing at a rate of 0.13 f.p.s. in a glass tube and at a temperature of 2.2°C was about 0.6 mg/l. This was the lowest TL_m value recorded.

The influence of water temperature on the TL_m's of oxygen concentration for naiads of _A. californica_ was most pronounced when the temperature was increased beyond 20°C. The influence of water velocity on the 24-hour TL_m's was most pronounced when the velocity was reduced below 0.03 f.p.s. An increase of velocity beyond 0.13 f.p.s. had little depressing effect on the TL_m's.

A limited amount of information on the oxygen tolerance of naiads of _A. californica_ collected in the vicinity of Corvallis, Oregon indicated that a considerable difference in tolerance exists between these naiads and those of _A. californica_ collected near Arcata, California. Limited data on the tolerance of naiads of _A. pacifica_ collected near Corvallis indicate that their tolerance may be about equal to that of _A. californica_ from the same area.

The mortality of _A. californica_ naiads from the Corvallis area was not much lower in the 24-hour tube experiments than in more prolonged tube experiments and in 14-day non-pollutional artificial stream experiments when oxygen conditions were comparable. Changes in the ventilatory movements of Acroneuria naiads suggest that the naiads were able to become acclimatized gradually to reduced concentrations of dissolved oxygen in the longer-term tests and thus survive indefinitely at concentrations which otherwise would have proved lethal.

Naiads of Acroneuria often performed series of ventilatory movements herein descriptively termed "push-ups." The rate or rhythm of these push-ups was expressed as the number per minute calculated on the basis of counts made over 20-second periods during which individual animals were performing push-ups continuously and at a steady rate. Counts were also made of the total numbers of push-ups performed continuously or intermittently during 5-minute periods.

The rate (rhythm) of push-up movements was influenced by temperature, as well as by the availability of oxygen, which evidently depends on both the concentration of dissolved oxygen and the velocity of the water. The peaks in the curves relating push-up rates of A. californica from the Arcata area to dissolved oxygen concentration were found to be at progressively higher oxygen concentrations as the temperature increased while the velocity remained constant, as were also the median limits of tolerance of reduced oxygen. At a constant level of dissolved oxygen, the velocity of the water markedly affected the percentage of all animals that engaged regularly in push-ups, as well as the average push-up rate of those animals engaged in these movements. Both decreased with increasing water velocity.

The mean rates of push-ups of naiads of A. pacifica were considerably lower than those of A. californica, regardless of the geographic origin of the naiads, at all tested dissolved oxygen concentrations, under constant conditions of water temperature and water velocity.

The total numbers of push-ups in 5-minute periods were also related to the concentrations of oxygen and the 24-hour TL_m's but not in the same way as were the push-up rates. The peaks in the push-up rate curves occurred at higher oxygen concentration than the 24-hour TL_m's for the same conditions, whereas the peaks in the curves for the 5-minute totals occurred at oxygen concentrations lower than the 24-hour TL_m's. Nearly up to the point of death, the 5-minute totals became steadily greater with decreasing concentrations of dissolved oxygen, because of more sustained ventilatory activity, and thus these totals seem to reflect, better than the push-up rates (i.e., rhythms), the low-oxygen stress to which the naiads were subjected.

The oxygen consumption rates of large naiads of *A. californica* from the Arcata area in standing water at 20°C. were dependent on oxygen concentration up to a level near the air saturation level and may be likewise dependent at higher concentrations. Although the animals were not apparently very active, the oxygen consumption rates which were measured may have been nearly the maximum rates or "active" rates for the conditions specified. These rates are in general agreement with rates reported for other similar forms.

A discussion is presented of some of the features, virtues, and limitations of artificial streams as tools for the study of the long-term tolerance of stream organisms to pollutional conditions, or for the identification of ecological factors in streams and evaluation of their importance. Also, critically discussed are the main short-comings of the reported studies with artificial streams. Possible improvements of apparatus and methods whereby these studies might have been made more productive of meaningful results and ways in which future studies having the same general objectives might be made more instructive are suggested.

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