THE THREESPINE STICKLEBACK, *GASTEROSTEUS ACULEATUS* L., AS A BIOASSAY FISH

by

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INTRODUCTION

The continual industrial expansion in the state of Oregon, as elsewhere on the Pacific Coast, has led many industries to consider locating plants on estuarine sites. At the present time it is not known to what extent the organisms inhabiting the marine and brackish waters will be affected by wastes from many of these industries. A study to determine the effects of kraft mill effluents to some marine organisms resident in Yaquina Bay was initiated in the spring of 1950 at Yaquina Bay Fisheries Laboratory of Oregon State College, Yaquina, Oregon.

A primary consideration was the selection of an organism that could be used as a standard bioassay animal for quick, reliable determinations of the relative toxicity of effluent samples. These samples were to be collected at different times and were to be stored under several conditions. Bioassays for periods of 24 hours or less were conducted on kraft mill effluents using many of the organisms available in the vicinity of the Yaquina Bay laboratory. Most of the animals initially tested were eliminated as possible standard test animals on the bases of size, lack of year around availability, and susceptibility to toxicities of pulp and paper wastes.

The threespine stickleback, Gasterosteus aculeatus aculeatus Linnaeus, and the amphipod, Anisogammarus confervicolus (Stimpson)[1], were finally selected as possible standard test animals and were used in several series of parallel bioassays. Although the two animals differed in their degree of tolerance to kraft mill liquors, they agreed quite closely in showing the relative toxicity of liquor samples. The threespine stickleback proved the superior test animal and was used for the remainder of the study, as the amphipod showed inconsistencies and was more tolerant of the wastes.

The purpose of this study was to determine the usefulness of the threespine stickleback as a bioassay fish, especially for marine or brackish water pollution studies. It is proposed that that concentration of waste necessary to kill 50 percent of the fish in 24 hours in water of 20 parts per thousand salinity and a temperature of 20° ± 2° C. be known as a "stickleback unit." The relative toxicity of various wastes can then be expressed in stickleback units.

There were two phases to this study. The first phase involved primarily the collection and analysis of unpublished data from experiments conducted by others at

[1] Identification was made by Dr. Ivan Pratt, Department of Zoology, Oregon State College.

the Yaquina Bay Fisheries Laboratory from January 1956 to June 1958. These data are from bioassays using sticklebacks and amphipods to test the toxicity of kraft mill black liquor and combined condensates in the proportion of one part black liquor to 99 parts condensates. The first phase of this study also included additional bioassays by the writer to verify or to supplement the above-mentioned data.

The second phase of this study was an investigation of the relative toxicity of kraft mill effluents to eggs, larvae, and very young sticklebacks.

Research for this study was conducted under the supervision of Professor Roland E. Dimick and Assistant Professor Wilbur P. Breese from January 1958 to March 1959. Preliminary work and data analysis were done at Oregon State College at Corvallis, while most of the experimentation was carried on at Yaquina.

LITERATURE REVIEW

Many fish and other fresh-water aquatic organisms have proven quite successful as indicators of the toxicity of kraft mill effluents and other materials discharged by industry into streams. Because of their economic importance, salmonids have been used quite extensively in this type of study although they do not adapt particularly well to laboratory conditions.

Haydu, Amberg, and Dimick (9, p. 545-549) employed chinook salmon, Oncorhynchus tshawytscha (Walbaum); coho salmon, O. kisutch (Walbaum); and coastal cutthroat trout, Salmo clarkii clarkii (Richardson) in toxicity bioassays in an attempt to pinpoint some of the toxic materials present in the kraft wastes. These bioassays were effective in determining the toxic range of some chemical constituents normally found in kraft mill wastes for each of the species of fish tested.

In a similar study, McHugh (18, p. 1-10) used coho salmon when testing total effluents for their toxic constituents. He found that important toxic components are contained in a steam-distillable, ether-soluble fraction of the wastes. Webb (22, p. 1-53) continued this work and determined that these toxic components described by McHugh are present in the condensates only, but that other toxic fractions are present in wash water.

Van Horn, Anderson, and Katz (20, p. 55-63) used four species of shiners belonging to the genera Notropis and Hyborhyncus and two types of fish food organisms, several aquatic insect larvae and Daphnia sp., in a study to determine which components of kraft mill wastes constitute the greatest hazard to fish life. They concluded that sulfides, mercaptans, resin acid soaps and sodium hydroxide present the greatest hazard.

Previously, Cole (4, p. 280-302) investigated the effects of kraft black liquor on perch, Perca flavescens (Mitchill); bluegills, Lepomis macrochirus Rafinesque; and largemouth black bass, Micropterus salmoides (Lacépède). He reported black liquor to be irritating to fish in concentrations of 1:500 or even higher, and to be definitely toxic in dilutions of 1:200 or less. He also reported that the toxicity of the liquor is decreased by aeration.

Considerably less work appears to have been done on the investigation of the effects of kraft mill effluents to marine or brackish water organisms, and relatively few fish or other marine organisms have been used as standard indicators of pollution.

The bream, Lagodon rhomboides (Linnaeus), was proposed as a standard test fish by Daugherty (5, p. 1029-1031) for determining the toxicity of industrial wastes

discharged into marine waters. He selected this fish mainly on its availability, distribution on the eastern coast of the United States, and low to medium tolerance to toxic materials.

Galtsoff et al. (8, p. 59-186) investigated the causes of the decline of oyster productivity and quality in the York River, Virginia. They reported that pulp mill effluents have a distinct physiological effect on oysters, particularly on their ability to accumulate glycogen.

Alderdice and Brett (1, p. 783-795) used sockeye salmon, O. nerka (Walbaum), to determine the safe concentration levels of kraft mill wastes in the estuary of the Somass River, British Columbia. They reported survival to be complete and independent of length of exposure below 4.8 percent concentration of effluent by volume in sea water of 20 parts per thousand salinity at 17.8° C. Upon considering the oxygen requirements for respiration, the net oxygen availability after effluent oxidation in the area, and the interaction of toxicity to the lowered oxygen, they concluded that the maximum effluent limit should be 2.5 percent concentration by volume.

The common killifish, Fundulus heteroclitus (Linnaeus), has also been used to some extent in bioassays, although it is probably too tolerant to many adverse water quality conditions to be a good standard

bioassay fish. It is euryhaline and found abundantly in many areas along the Atlantic coast. Wilber (23, p. 1-13) used this species when testing octamethyl pyrophosphoramide. He indicated this fish was selected because of its hardiness, since his primary aim was not to ascertain minimum lethal concentrations but to determine the comparative susceptibility of an estuarine fish to an organic phosphorus compound.

Much of the work using sticklebacks in bioassays appears to have been done by Jones. In experiments using heavy metal salts, he reported that the toxicities of zinc, copper, and lead were reduced when calcium was added (12, p. 394-407) (13, p. 425-437). The alkalis and alkaline earths enter the body and act as true poisons. All other metal ions bring about death by precipitating gill secretions and causing asphyxiation. The metals of low solution pressure whose ions actively enter into combination with other ions or compounds show the greatest toxicity since they will most readily precipitate the gill secretions. When the gas exchange in fish is impeded in this way, the respiratory centers are stimulated as the tissues produce carbon dioxide, which in turn increases the opercular movements, and the fish dies of fatigue and asphyxiation.

When sticklebacks were subjected to solutions of chloroform, sodium cyanide, and sodium sulfide (14, p. 298-311), an anesthetic effect was produced. Less oxygen was consumed, and opercular movements showed a corresponding decrease. This is due to the decreased ability of the tissue to use oxygen, and less carbon dioxide is produced. There was no interference of gaseous exchange.

Reaction experiments by Jones (15, p. 22-34) (16, p. 403) showed that sticklebacks will enter water with low dissolved oxygen, and when entering such water, their movements will increase until they chance to get into oxygenated water again. When the fish were free to move from tap water into a toxic solution, they avoided water with a pH of less than 5.6 or higher than 11.4. To ammonia, sticklebacks react negatively, or will not enter 0.04 and 0.01 N solutions, but act positively, or will enter solutions of 0.001 and 0.0001 N. To lead nitrate they react positively to 0.04 and 0.01 N solutions, and negatively to 0.004 down to 0.00002 N solutions.

METHODS AND MATERIALS

Threespine Stickleback

The threespine stickleback is a small euryhaline fish inhabiting most coastal waters, bays, and slower more coastal streams in the temperate and cooler areas of the Northern Hemisphere. It is quite abundant throughout most of its range, and is of only little or no economic value.

There is considerable morphological variation in the threespine stickleback, especially with regard to the presence and numbers of scutes, which has given rise to much confusion in classifying the fish. Most sticklebacks with three spines are now classified as Gasterosteus aculeatus, and localized variations are considered subspecies or races. Throughout most of its range the stickleback occurs in two forms, one inhabiting marine and brackish water, and the other inhabiting only fresh water.

In Oregon the marine form, Gasterosteus aculeatus aculeatus Linnaeus, inhabits most coastal areas, and particularly bays and estuaries, and will enter fresh water occasionally. The other form, Gasterosteus aculeatus microcephalus (Girard), remains in fresh water throughout its entire lifetime and will normally be found in lower, slow-moving streams.

The breeding seasons and occasionally the spawning areas of the two forms may overlap, and apparently a few marine fish crossbreed with fresh-water fish, but most of the sexually mature marine sticklebacks taken from Yaquina Bay were in water of about 30 parts per thousand salinity. In one area, however, both G. a. aculeatus and G. a. microcephalus were taken at the same time from water with a salinity content of 1.4 parts per thousand. Koch and Heuts (17, p. 253-266), working in Belgium, found that the marine form will enter fresh or nearly fresh water to spawn and may even become intolerant to high salinities during the breeding season. In Yaquina Bay the sexually mature marine sticklebacks found commonly in water of about 30 parts per thousand salinity were apparently not adversely affected by this salinity, but when eggs were artificially fertilized in the laboratory a much better hatch was obtained at lower salinities.

Both G. a. aculeatus and G. a. microcephalus have three dorsal spines, one pair of ventral spines, and an anal spine, all of which are erectile. Their pectoral fins are large and truncate. No scales are present, but they have a row of bony plates, or scutes, on each side extending caudad from the region of the pectoral fins. These scutes in the marine form usually number about 30 to 35 on each side and extend onto the caudal peduncle

where they form a keel. In Oregon these scutes may be used as a quick identification character between subspecies, as G. a. microcephalus ordinarily has six or fewer scutes anteriorily and no keel, which G. a. aculeatus has a keel and normally 30 or more scutes on a side, Figures 1 and 2. An occasional fish resembling the fresh-water form was found in salt water, but these may have been subspecific hybrids.

The differences in scute number and other morphological variations may be correlated with inherent physiological differences. Heuts (10, p. 89-102) found this to be true for different forms of the threespine stickleback in Belgium (Types A and B). Type A, the fresh-water form, is adapted to high temperatures and low salinities. Type B, the marine form, is adapted to low temperatures and high salinities. There is some hybridization, but the hybrids resemble one group or the other physiologically and are variable morphologically.

Sticklebacks found on the Pacific coast of North America appear to differ in several respects from those found on the east coast or in Europe. Vrat (21, p. 252-256) observed that the breeding season is longer by about two months on the coast of central California, and that the male fish chose to build their nests in depressions in mud or sand rather than in weeds.

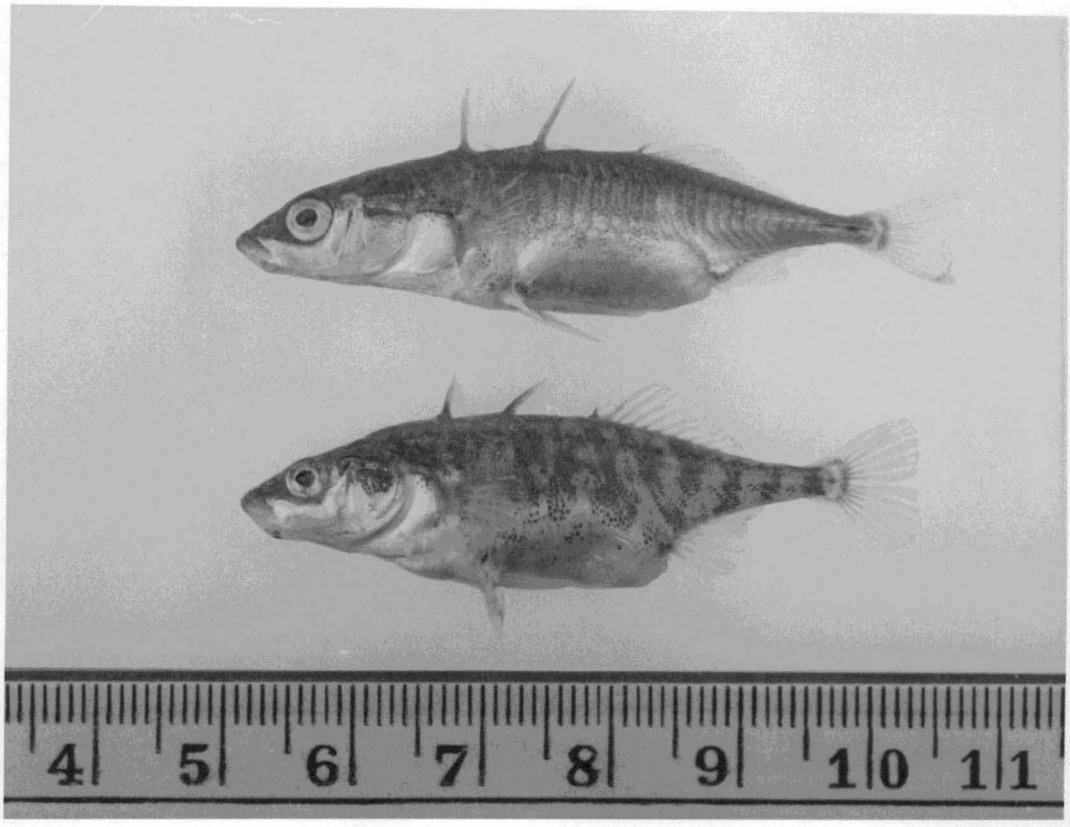

Figure 1. Left side view of gravid female sticklebacks showing differences in the marine form, Gasterosteus aculeatus aculeatus L. (top), and the fresh-water form, G. a. microcephalus (bottom). Note longer dorsal spines, no mottling, and scutes extending onto the caudal peduncle on the marine fish.

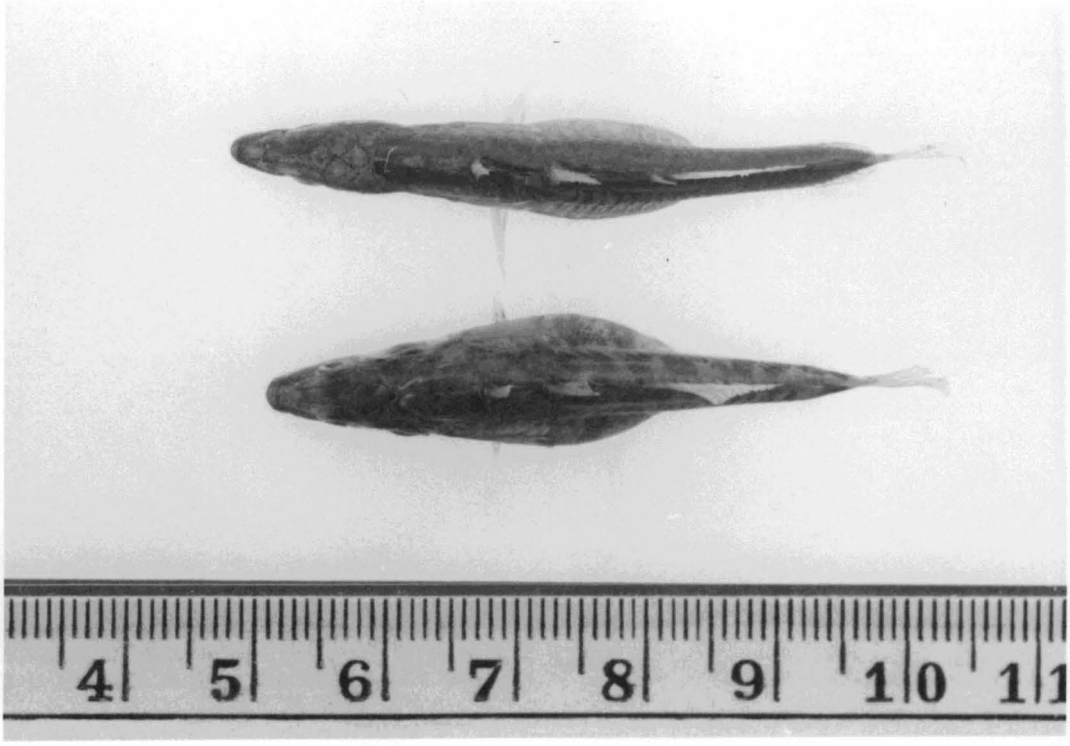

Figure 2. Dorsal view of gravid female sticklebacks showing differences in the marine form, Gasterosteus aculeatus aculeatus L. (top), and the fresh-water form, G. a. microcephalus (bottom). Note longer ventral spines, more slender body, and scutes forming a keel on the caudal peduncle of the marine fish.

The marine stickleback possesses many characteristics that are considered desirable for a bioassay fish. It is small in size, with a maximum length of about 3 inches; it is quite abundant throughout most of its range; and it is easily taken without expensive or complicated collecting gear. Small sticklebacks ranging in size from 25 to 40 mm. total length can be collected in abundance throughout the year near the Yaquina Bay Fisheries Laboratory using only a short-handled dip net. Sticklebacks can be held in tanks in the laboratory for several weeks if necessary without significant mortality, and when placed in small containers for bioassays they are not easily excited by the presence of people working nearby. The stickleback acclimates readily to salinity differences and is neither overly resistant nor particularly intolerant to the toxic materials tested thus far. The widespread distribution of this fish in the Northern Hemisphere makes it particularly desirable for use as a standard test animal, especially in estuarine waters.

Many studies have been published on the distribution, reproduction, breeding behavior, food habits, parasites, morphology and physiology of stickleback fish. No attempt will be made here to list all of the literature pertaining to these studies. However several papers, especially the more recent ones, are outstanding and should be mentioned.

Iersel (11, p. 1-159) held sticklebacks in the laboratory for several months and made comprehensive studies of their normal behavior, breeding behavior, and parental care. He has also included an extensive review of many previous studies. Craig-Bennett (5, p. 197-279) and, more recently, Baggerman (2, p. 105-318) have published on the factors responsible for timing of migrations and breeding cycles of the stickleback. Much of both studies was concerned with the physiological changes connected with gonadal maturation, migration and breeding. Pickford (19, p. 37-474) and Brown (3, vol. 2, p. 169-329) have also made reference to physiological studies on the stickleback.

Collection and Care of Fish

All fish used in this study were collected locally from small channels and potholes in tidal-flat sloughs adjacent to Yaquina Bay. The tidal flats are covered by salt grasses and weeds, and are extensively cut by small channels and potholes that flood at high tide and drain to only a few inches of standing water at low tide. Since sticklebacks may move from one area to another with the high tides, they can be taken from many of the same areas each time collections are made.

Experimental specimens were collected from shallow-water areas at low tide as they were needed and placed in a 250-gallon wooden tank of sea water having a salinity of approximately 20 parts per thousand and a temperature of 20° C. They were allowed to acclimate to these conditions from one to several days before being used in bioassays. Sticklebacks acclimate rapidly to salinity and temperature changes, and a longer acclimation period was deemed unnecessary, based on previous tests at the laboratory. If the fish were held more than three days, they were fed chopped cockle clams, and they were generally not held more than two weeks.

Kraft Mill Effluents

The kraft pulp mill liquors used in these experiments came from a single Northwest pulp and paper mill. Three types of liquors were tested: black liquor (spent cooking liquor), combined condensates, and wash water. Condensates and wash water were collected in rubber-stoppered, small-mouthed five-gallon jugs. Black liquor was collected in one-gallon jugs. The liquors were numbered consecutively as they were collected, as a means of keeping the samples separate in the records. Black liquor and combined condensates samples were collected periodically from December 195 to September 1958, and these samples will be referred

to as kraft #1 through kraft #26. The first wash water samples were collected on June 20, 1958, and will be referred to as wash water #1 through wash water #6. Each batch of liquor was bioassayed upon arrival at the laboratory to determine its relative acute toxicity. The toxicity for each batch was then recorded in stickleback units, or that concentration of liquor calculated to kill 50 percent of the fish in 24 hours in water of 20 parts per thousand salinity and a temperature of 20° ± 2° C.

Throughout this paper, the kraft pulping wastes will also be referred to as "effluents" or "wastes."

Black Liquor. This is a highly alkaline, black liquid forced from the digesters after the cooking cycle. It contains the spent cooking chemicals, which are principally sodium hydroxide and sodium sulfide, employed to break down the wood into usable fibers. Black liquor is not discharged as a waste. It is sent to the recovery plant, where it is evaporated and burned. In this way, the chemicals in the ash are reusable, and the heat of combustion is recovered. A small amount of black liquor may be washed from the pulp fibers and be discharged in the final-stage wash water.

Combined Condensates. These are the condensed and "non-condensable" waste materials from the recovery plant, together with condensed liquids that are forced out of the

digesters with the steam during the cooking cycles. Most condensates are light-colored and mildly alkaline, with only a trace of total solids. Throughout this paper these will be referred to as "combined condensates" or "condensates."

Final-stage Wash Water. When pulp is blown into the wash vats from the cookers, it still contains a considerable amount of black liquor. Most of this black liquor is removed by a series of washings. The water for this series of washings is recirculated until it is concentrated enough to be sent to the recovery plant. The final-stage wash water is too dilute in chemicals for economical recovery, and it is discharged as a waste.

At the outset of this study, it was thought that the toxicity of wash water might be similar to that of dilute black liquor and that diluted black liquor could be used in bioassays and make the transportation of large quantities of wash water unnecessary. Subsequent bioassays, however, indicated that wash water may have quite different toxicity characteristics than dilute black liquor.

Bioassay Defined

A bioassay is a test using the response of living organisms to artificial environmental situations usually under controlled laboratory conditions. In the acute

toxicity bioassays reported in this study, tests were run for a period of 24 hours, and the response noted was death of the fish. The relative acute toxicity of wastes is designated by the calculated median tolerance limit (TL_m). The 24-hour median tolerance limit is that concentration at which 50 percent of the fish are expected to die (or to stay alive) in 24 hours. The 50-percent death concentrations, or TL_m's, were determined graphically using the method suggested in Doudoroff et al. (7, p. 1380-1397).

General Bioassay Methods

All acute toxicity bioassays were conducted for periods of 24 hours or less at a temperature of 20° ± 2° C. and a salinity of 20 parts per thousand. Fish were acclimated to this temperature and salinity for at least one day prior to being used in bioassays. One-gallon glass pickle jars with a water surface area of approximately 182 square centimeters were used for all bioassays except those for eggs or larval fish. The jars were filled to 2000 ml. with prepared wastes diluted to various concentration levels, and five fish or ten amphipods of uniform size and apparent good physical condition were placed in each jar. The waste dilutions were changed after 12 hours to prevent dissolved oxygen depletion by the fish and the wastes. Four jars of prepared solution and twenty fish were used at each waste

concentration tested. At the end of each bioassay all fish were discarded.

The solutions to be tested were prepared from the wastes immediately before bioassays were to be conducted by mixing a stock solution of one part black liquor to 99 parts combined condensates. Dilutions were made in well-aerated marine water adjusted with fresh water to a salinity of 20 parts per thousand.

To determine the range of concentrations of liquors for the 24-hour bioassays, accelerated tests were conducted using 15 percent of prepared waste in each of four jars with five fish per jar, or a total of 20 fish. As each fish died, its time of death in minutes and its total length in millimeters were recorded. The last fish usually died within four hours. The times it took for the first, tenth, and last fish to die were then used as indications of the toxicity of the waste and formed the basis of the concentrations selected for the 24-hour bioassays.

Procedure for Acute Toxicity Bioassays. Both 24-hour bioassays and accelerated short-term bioassays were employed to determine the effects of storage upon the toxicity of kraft liquors, the relative acute toxicity of the several samples of kraft liquors, and the relation of fish size to toxicity tolerance. The majority of the testing was with one part of black liquor to 99 parts condensates

on the assumption that such a ratio would approximately duplicate the toxicity of the combined discharge of condensates and wash water. Some preliminary experiments were made separately in black liquor or in wash water.

Effect of Storage on Liquors. To ascertain if kraft mill effluents used in bioassays decrease in toxicity when kept under different storage conditions, a single sample (kraft #10) was collected from the pulp mill in several glass containers on December 28, 1956. These effluents consisted of black liquor, held in one-gallon jugs, and combined condensates, held in five-gallon glass jars. Upon arrival at the laboratory, the original sample was bioassayed to establish the toxicity to sticklebacks and amphipods. The sample was then divided into several lots or subsamples and stored as follows: (1) closed glass containers refrigerated at 1° to 2° C.; (2) closed glass containers held at room temperatures, 18° ± 4° C.; (3) open glass containers held at room temperatures, 18° ± 4° C.; (4) closed glass containers held at outside temperatures, 2° to 15° C.; and (5) held frozen in waxed paper milk cartons. Condensates and black liquor were stored separately and mixed in the proportion of one part black liquor to 99 parts condensates immediately prior to being used in bioassays. The condensates from a single container

were used for a series of experiments until the container was emptied, and then a full container was opened.

Four groups of bioassays were conducted on kraft sample #10 over a period of 25 days. Fish and amphipods were each used in two series of bioassays, one series in which no air was bubbled through the test solutions, and the other series in which the solutions were aerated. Both the aerated and the non-aerated solutions were changed at 12-hour intervals.

Test periods varied with each stored waste. Refrigerated wastes in closed containers were tested after 2, 5, 8, 11, and 18 days storage; wastes stored at room temperature in closed containers were tested after 3, 6, 9, 16, and 23 days storage; wastes stored at room temperature in open containers were tested after 4, 7, 10, 17, and 24 days storage; wastes stored in a frozen condition were tested after 19 and 25 days storage; and wastes stored at outside temperatures in closed containers were tested only after 20 days.

On February 13, 1957, kraft sample #11 was collected and used to determine, if possible, the effects on the acute toxicity of condensates when they are stored in full, sealed containers. Upon arrival at the laboratory, the liquor was tested for its original toxicity. The condensates sample was then distributed into one-gallon jugs

which were filled to the top and then sealed, except the portion of the condensates sample to be frozen, which was put in one-quart polyethylene acid bottles and sealed. The condensates were then divided into lots and stored refrigerated, 1° to 2° C.; at room temperatures, 18° ± 4° C.; at outside temperatures, 2° to 15° C.; and frozen. A new container of condensates was opened for each bioassay and not used in any subsequent tests. All bioassay procedures were similar to those of the non-aerated tests of kraft sample #10 described above.

The subsamples stored under different conditions were tested periodically. Refrigerated samples were tested after 5, 12, 19, and 40 days; room temperature samples after 6, 13, 20, and 41 days; outside temperature samples after 7, 14, 21, and 42 days; and frozen samples after 2, 8, 15, and 48 days.

Toxicity of Black Liquor. There was some evidence from preliminary tests that black liquor does not have the identical toxicity properties of wash water. To determine, if possible, what some differences might be, a series of exploratory tests was conducted with black liquor. In addition, bioassays with combined condensates and black liquor for periods of 24 hours were conducted. One series of tests was with dilutions made from wastes of one part black liquor and 99 parts condensates, while the other

series contained only condensates dilutions.

No 24-hour bioassays were conducted on black liquor alone, since a precipitate was formed when solutions were mixed in salt water at concentrations high enough to kill over 50 percent of the fish. The precipitate might possibly have contained much of the toxic material or could have physically interfered with the respiration of the fish, thus making the bioassays meaningless. Short-term bioassays were conducted on black liquor, however, in parallel with bioassays on 15 percent solutions of condensates, one part black liquor to 99 parts condensates, and wash water. The concentration of the black liquor solution to be used was arbitrarily derived from a measurement of the total solids by weight in wash water and black liquor. The total solids in 100 percent wash water were found to be 0.2 percent, and the sample of black liquor had a total solids content of 15.3 percent. As a possible comparison of the black liquor and wash water, a stock solution was made by diluting black liquor with fresh water until the total solids were 0.2 percent by weight, and then 15 percent of this stock solution was used in these short-term bioassays. When the stock solutions of black liquor, 100 percent wash water, 100 percent condensates, and 1:99 black liquor to condensates were diluted to 15 percent by volume with salt water, the

pH in these solutions varied from 7.7 to 8.9. In order that more accurate comparisons might be shown, experiments were made in duplicate. One series of tests was at the pH of the mixtures, and the other series was adjusted to pH 7.4 with hydrochloric acid.

Another set of short-term bioassays was conducted using approximately 15-percent mixtures of black liquor and condensates in the following proportions: 1:99, 2:99, and 4:99. The quantity of condensate was held constant at 297 ml. in 2000 ml. of solution so that any appreciable variance in toxicity would be attributable to the difference in black liquor. In all bioassays using 15 percent dilutions, the times of death were recorded for each fish, and no median tolerance limits were calculated.

Wash Water Bioassays. The wash water was erroneously believed to be relatively non-toxic when this study was undertaken, but since then experiments at the Pacific Cooperative Water Pollution Laboratory at Oregon State College have indicated that wash water may have at times a relatively high toxicity to fish when the pH is adjusted to about neutral. Since it was not known what effect salinity would have on the toxicity of wash water or how the toxicity would vary with changes in pH, all tests on wash water were exploratory in nature.

One series of tests was conducted to determine the 24-hr. TL_m with the pH not adjusted. A second series was conducted to find the 24-hr. TL_m using wash water with the pH adjusted with hydrochloric acid to that of the dilution water, about pH 7.4. To determine if concentrations of wash water with the pH adjusted to 7.4 were more toxic than the same concentrations at higher or lower pH levels, a set of tests was arranged holding the wash water constant at 5 percent by volume and varying the pH from 4 to 10 with hydrochloric acid and sodium hydroxide respectively.

To check the possibility that hydrochloric acid or sodium hydroxide or their dissociated ions produce a toxicity in sea water, these compounds were used to vary the pH of water from 4 to 10 in a series of jars. These solutions were then tested with sticklebacks for toxicity.

Acute Toxicity versus Size. A single experiment using fish from 20 to 65 mm. total length to determine the variation in tolerance of size groups to kraft mill wastes was conducted with kraft sample #10. At the end of 12 hours the number of dead fish and their sizes were recorded, and at the end of 24 hours the number of dead fish and the sizes of both the dead and live fish were recorded.

Procedure for Bioassays Using Larval Fish. Bioassays were performed to determine the tolerance of stickleback embryos and larvae to kraft mill wastes. Eggs and sperm

were taken from sexually mature fish which had been collected near the laboratory, and the eggs were fertilized artificially. The eggs and larval fish were held under laboratory conditions until they reached the desired developmental stage for testing.

Fertilization Procedures. The methods used to collect eggs and sperm from adult fish, to fertilize the eggs, and to hold the embryos and larval fish in the laboratory were developed before the toxicity study was started. The fertilization procedure is as follows:

1. Eggs were stripped from the female directly into sea water of 20 parts per thousand salinity or into toxic solutions in small dishes. Four-inch stacking dishes containing 250 ml. of water were found to be adequate for this purpose. If the eggs are to be placed in more than one solution, they can be stripped a few at a time and distributed as needed.

2. Sperm were taken by removing the testes from the male and macerating them in a small amount of water. An eyedropper was used to transfer sperm into the water containing the eggs.

3. Eggs were either well separated or were aerated during development to prevent mortalities from low dissolved oxygen.

All eggs or larval fish used in a single bioassay were from one female. When the fertilized eggs had developed to the desired stage, they were divided among five stacking dishes each containing 250 ml. of water or waste solution. The eggs or larval fish were left in the waste solutions for 24 hours and then were transferred to clean marine water of 20 parts per thousand salinity where they were allowed to continue developing.

The liquors used were the same as those employed in bioassays on older fish. Most of the bioassays were conducted in solutions made from a stock solution of one part black liquor to 99 parts condensates.

Since aeration reduces the toxic effect of these liquors, the solutions were not aerated, and the solutions were not changed after 12 hours as they were with bioassays using older fish 25 to 40 mm. long. All bioassays were at 20° ± 2° C., and a final dilution water of 20 parts per thousand salinity was used.

Toxicity Bioassays. Nine bioassays were conducted with eggs and fish ranging from freshly fertilized eggs to juvenile fish 14 days after fertilization. Ages were recorded from time of fertilization.

The tolerance to pH by fish was tested in parallel bioassays conducted for 24 hours. One series of tests was in waste solutions of one part black liquor to 99 parts

condensates. The other bioassay series was performed in water of 20 parts per thousand salinity with no liquors added, but with the pH adjusted in each of five dishes to correspond with the pH's in the waste dilutions.

To determine if the age at hatching is an important factor in fish tolerance to kraft liquors, parallel bioassays were conducted on two groups of fish hatched from the same lot of eggs. One bioassay was with fish hatched during the first 24 hours of the 2½-day hatching period. The second bioassay was with fish hatched the last 24 hours of the hatching period. Bioassays on both groups were performed one day after the last fish hatched. All fish were about eight days old from fertilization at this time.

EXPERIMENTAL RESULTS

Acute Toxicity Bioassays

The 24-hr. TL_m of sticklebacks in kraft sample #10 tested a few hours after mill collection was 1.74 percent by volume in non-aerated solutions and 2.5 percent in aerated solutions. For amphipods, the 24-hr. TL_m of the sample was 3.25 percent in non-aerated solutions and 4.95 percent in aerated solutions. These four initial bioassays indicated that amphipods were more tolerant to kraft mill wastes than were sticklebacks and that aeration of the testing solution during the bioassays reduced the toxicity. These relative differences held true throughout the series of tests on kraft mill sample #10. The differences in tolerance did not always hold true for other samples collected later, however.

Kraft mill sample #10, after storage in the laboratory, lost some toxicity regardless of the storage conditions. Inspection of the data in Tables 1, 2, 3, and 4 indicates that the most rapid loss in toxicity was in samples stored in open containers, and that storage temperatures between 1° and 20° C. have very little, if any, effect on the rate of loss. The losses throughout the storage periods were about the same for all samples stored in closed containers, while the losses of toxicity in open

containers was about 50 percent greater. For example, wastes stored at room temperatures and tested in non-aerated bioassays with sticklebacks had an original 24-hr. TL_m of 1.7 percent by volume, and after storage for 23 days wastes stored in closed containers had a TL_m of 3.8 percent, and wastes stored for 24 days in open containers had a TL_m of 6.5 percent. The other series of tests on this sample showed comparable toxicity losses.[2]

The liquor refrigerated at 1° to 2° C. demonstrated a gradual loss in toxicity during the storage period in all four series of bioassays. The rate of toxicity loss was uniform, and the concentrations of liquor required to produce a 24-hr. TL_m could be predicted in most cases even after prolonged periods of storage. Table 1 shows that after eleven days storage, the toxicity to sticklebacks had decreased from TL_m 1.7 percent to 4.33 percent in non-aerated bioassays and from 2.5 percent to 6.8 percent in aerated bioassays. Similarly, when amphipods were used the toxicity decreased during the same period of time from 3.25 percent to 3.4 percent in non-aerated bioassays and from 4.95 percent to more than 10 percent in aerated bio-assays.

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[2] Since the 24-hour median tolerance limit is expressed as percent by volume of waste, the TL_m will increase as the toxicity decreases.

After 18 days wastes from full containers were more toxic than wastes stored for 11 days in partially full containers. For example, the 24-hr. TL_m percents for sticklebacks in non-aerated bioassays were 4.33 at 11 days and 4.0 at 18 days.

Data from Table 2 indicate that samples of liquor stored at room temperatures in stoppered containers lose toxicity to about the same extent as do refrigerated samples. After storage for nine days at room temperatures, the 24-hr. TL_m's for stickleback fish were 3.8 percent in non-aerated and 8.0 percent in aerated bioassays, and for amphipods were 4.41 percent and 8.81 percent for non-aerated and aerated bioassays. These concentrations correspond fairly closely to the TL_m's of wastes refrigerated for eight days, which were 3.72 and greater than 6.0 percent for sticklebacks and 5.0 and 7.0 percent for amphipods.

Wastes stored in closed containers at outside temperatures were tested after 20 days only. The small loss in toxicity may be attributable to the wastes having been stored in full, stoppered containers. The 24-hr. TL_m's went from 1.7, 2.5, 3.25 and 4.95 percent to 2.45, 3.7, 3.0 and 6.0 percent respectively. The bioassay with amphipods in non-aerated solutions is one of the two times

during the tests on kraft mill sample #10 in which the wastes indicated no loss in toxicity during storage.

Table 3 shows that liquors stored in unstoppered containers at room temperatures lost toxicity rapidly. For example, the TL_m increased from 1.7 percent to 6.44 percent after storage for ten days when tested with sticklebacks in non-aerated solutions.

When wastes were frozen in unsealed milk cartons, the losses in toxicity appeared to be considerable. These wastes were tested in several bioassays, but due to toxicity losses being greater than expected, a TL_m could be estimated from only one bioassay. At 25 days storage, the TL_m was 5.6 percent when tested with sticklebacks in non-aerated bioassays, Table 4.

Kraft sample #11 was divided into subsamples and stored in full, stoppered one-gallon jugs or frozen in full one-quart polyethylene bottles. All bioassays were run on non-aerated solutions.

As can be seen in Table 5, very little, if any, loss in toxicity to sticklebacks appears to occur with storage up to 40 days, except in that portion of the sample that was frozen. The frozen wastes in stoppered polyethylene bottles lost toxicity when stored. The initial TL_m with sticklebacks was 2.2 percent, 6.0 percent after 19 days, and 6.33 percent at 40 days.

When amphipods were used as the test animals, only a small decrease in toxicity was shown with storage except in the case of frozen wastes, where a more rapid loss occurred. The median tolerance limits increased from 2.25 to 3.43 percent with refrigerated samples in 40 days; to only 2.83 percent with room temperature samples at 41 days; to 3.67 percent with outside temperature samples at 42 days; and to 5.85 percent with frozen samples in 48 days. It can be seen from Table 5 that most of these losses in toxicity were gradual throughout the storage period.

Toxicity of Black Liquor. There was no significant difference in the results obtained from 24-hour stickleback bioassays using condensates only and those tests in which one percent black liquor was added to the dilutions. Kraft sample #23 showed a slight decrease in toxicity when the one percent black liquor was omitted. A TL_m of 3.83 percent was obtained without black liquor and a TL_m of 3.27 percent with black liquor. Kraft sample #24 showed a very slight increase in toxicity when black liquor was omitted, the TL_m being 3.27 percent without black liquor and 3.38 percent with black liquor, Table 6.

Black liquor was clearly shown to contribute some toxicity in accelerated bioassays with concentrations using about 15 percent wastes. When condensates were held constant at 297 ml. per 2000 ml. of solution and the black

liquor was added at rates of 3 ml., 6 ml., and 12 ml. respectively, the survival times were reduced accordingly. With 3 ml. of black liquor in the solution, only 11 out of 20 fish died in 210 minutes; with 6 ml. of black liquor, all 20 were dead in 210 minutes; and with 12 ml. of black liquor, all were dead in 155 minutes, Table 7.

Table 8 shows that 15-percent waste solutions which include one percent black liquor are more toxic than the same waste concentration without any black liquor. This held both in samples that were adjusted to pH 7.4 and in samples left unadjusted. The average time of death of fish in wastes including black liquor was 64.5 minutes at a pH of 7.4, and 65.5 minutes at a pH of 8.7. The average times of death in condensates solutions were 250 minutes with the pH adjusted to 7.4 and 221.5 minutes with the pH left at 7.7. As can be seen from these data, pH had only a slight effect on the toxicity of these solutions to sticklebacks, Table 8.

When solutions of black liquor and of wash water containing equal total solids were bioassayed, the black liquor was considerably less toxic. In such concentrations the black liquor with pH 8.6 killed no fish in 24 hours, while wash water with pH 7.9 produced a 100-percent kill in 24 hours. After the 15-percent solutions were adjusted to pH 7.4, black liquor killed 70 percent of the fish in 24

hours, and wash water produced a 100-percent kill in about three hours. It appears from these data that both wash water and black liquor contain a component that becomes toxic, or more toxic, when the pH is lowered to 7.4, Table 8.

Wash Water Bioassays. A series of tests showed wash water to have TL_m's of about 25 to 30 percent by volume when the pH was left unadjusted at approximately 9.0 to 9.6. But when the pH was adjusted to pH 7.4, or slightly above neutral, the toxicity increased, and the TL_m's were about 2 percent in most bioassays, Table 9.

Five percent wash water exhibited a relatively high degree of toxicity to sticklebacks when pH values were below 8.0. In 5-percent wash water dilutions with pH 5.5 through 8.0, all of the fish died in 24 hours. The toxicity was less in 5-percent dilutions with the pH adjusted to 4.5 or 5.0, with 40 percent of the fish remaining alive at 24 hours. From pH 8.5 through pH 10.4, wash water had a low toxicity to sticklebacks; in this range in pH no fish died in 24 hours, Table 10.

The toxicities of hydrochloric acid and sodium hydroxide were tested in solutions of 20 parts per thousand salt water with the pH adjusted from 4.0 to 10.0. The pH of the salt water was 7.4 initially; hydrochloric acid was used to lower the pH to 7.0 or below, and sodium

hydroxide was used to increase the pH to 8.0 or above. In 24-hour bioassays fish tolerated pH 6.0 through pH 10.0 with no mortality. At pH 4.0 there was 80 percent mortality, and at 5.0 there was 20 percent mortality.

Toxicity versus Size. Fish ranging in total length from 25 to 60 mm. varied only slightly in their tolerance to Kraft wastes in 24-hour bioassays. The fish around 40 mm. to 44 mm. appear to be slightly more tolerant than the other size groups, while fish under 25 mm. or over 60 mm. long appear considerably less tolerant than the other groups, Table 11.

Larval Fish Bioassays

Developing stickleback eggs and larval fish displayed a much greater tolerance to kraft mill wastes than did juvenile fish that had absorbed their yolk sacs. The 24-hr. TL_m never dropped below 7.0 percent concentration of waste by volume in any test with eggs or with fish retaining their yolk sacs, while fish fourteen days old, or approximately one week from hatching, displayed a TL_m of 3.45 percent. The 24-hr. TL_m on these 14-day-old fish corresponded quite closely to the TL_m of older fish 25 to 40 mm. long, Table 12.

Fish the same age from fertilization, but different ages from time of hatching, are about equally tolerant of

kraft wastes. Two groups (one day old and three days old) from the same brood had the same TL_m of 8.2 percent.

The rate of development in any single batch of eggs was not constant, and did not appear to determine when the fish would hatch. Eggs hatched at various stages of development over a period of about two and one-half days. Some fish still had large yolk sacs, while other individuals hatched with their yolk sacs mostly absorbed. The most tolerant fish at the higher waste concentrations were those individuals showing the least development and having the largest yolk sacs.

No toxicity to one-day-old fish was demonstrated in 24-hour bioassays by water adjusted to the pH's 8.3, 8.55, 8.9 and 9.1, which were the pH's produced by waste solutions of 5, 10, 20, and 30 percent by volume.

DISCUSSION

The threespine stickleback was selected for this study because of its small size, availability in large numbers, and tolerance to rapid salinity and temperature changes. The experiments reported previously indicate also it is a reliable bioassay organism, at least for kraft pulping wastes.

The relative toxicity of the different samples of kraft mill wastes was determined by use of the threespine stickleback and was recorded in "stickleback units." Using this data, dilutions of liquor based on the stickleback unit of toxicity were made and used in continuous-flow experiments not reported in this thesis. These continuous-flow experiments covering extended periods of time are part of a program to study the overall effects of kraft mill wastes on oysters and other marine fauna.

The liquor samples collected from the kraft pulp mill and tested for toxicity in these experiments were grab samples and were not composites of the varying effluents produced during a complete cooking and blowdown cycle of the digesters. This sampling defect and minor changes in the mill operations at various times probably caused the differences in the composition and toxicity of liquor samples used. For example, there was a difference of

coloration of condensates in the subsamples of kraft sample #25. One portion that was lighter in color was also more toxic.

Some of the toxic fractions in condensates are probably volatile at above-freezing temperatures, which may account in part for the loss in toxicity of the condensates when stored or when aerated during a bioassay. The data indicate that there is also a loss in toxicity when wastes are stored in a frozen condition. This loss in toxicity in frozen samples may also be partly due to the escape of highly volatile compounds. In addition, biological or chemical decomposition of the toxic elements may account for some of the observed decreases in toxicity in stored wastes.

The cause for the changes in toxicity of wash water with the raising or lowering of pH is beyond the scope of this study. Above pH 8.0 the wash water had a low toxicity level, but at pH 8.0 or below the toxicity is increased markedly.

The toxicity of black liquor cannot be correlated to the toxicity of wash water. When black liquor was diluted with water until its total solids were equal to that of the wash water, the black liquor solutions had a much darker color, had a greater amount of precipitates when mixed with sea water, had a lower pH, and had a lower

toxicity. Similarly, if both were adjusted to the same pH, differences in color, total solids and toxicity were present; and if both were adjusted to the same color, the pH, toxicity, and total solids differences were still present.

Embryos and larval stages of sticklebacks are quite tolerant to kraft mill wastes. Sensitivity to the kraft liquors appears to increase at about the time the fish loses its yolk sac and first begins to pump water past its gills. Data do not indicate the reasons for the toxicity of kraft mill wastes to fish. It may be that the kraft mill liquors adversely affect the gaseous transfer in the gills, causing the fish with functional gills to die more readily than those fish that are still obtaining oxygen by diffusion through the yolk sac, or that kraft mill toxicants can only enter the body of the fish by means of gill membranes. Apparently kraft wastes do not permanently injure sticklebacks, since the fish surviving a liquor concentration will ordinarily recover completely if placed in clean water.

SUMMARY

1. The threespine stickleback was tested for suitability as a bioassay organism in a series of tests evaluating its sensitivity to kraft mill effluents. Bioassays were made using various kraft mill waste concentrations in water of 20 parts per thousand salinity at a temperature of 20° C. Stickleback fish were found suitable for use in toxicity determinations and were established as standard test animals, and the "stickleback unit" was adopted as a measurement of the relative acute toxicity of each kraft mill sample.

2. Black liquor and combined condensates were tested together for loss in toxicity after being stored. Storage caused a reduction of toxicity of these wastes which was independent of temperature. The loss in toxicity was reduced when wastes were held in full, stoppered containers.

3. Black liquor was used in place of wash water in bioassays with condensates. Tests indicated that dilute black liquor and wash water are not similar and that black liquor should not be used to replace wash water in bioassay procedures.

4. Wash water was tested to determine changes in toxicity at different pH levels. The toxicity was at a low level when the testing solutions were held at pH's

above 8.0, but when the pH's were lowered to 8.0 or below, the toxicity increased markedly.

5. Fish with total lengths of 25 to 60 mm. were relatively consistent in their tolerance to kraft mill wastes and were used in all toxicity bioassays.

6. Embryonic and larval sticklebacks displayed about twice the tolerance to kraft wastes as did older fish 25 to 40 mm. long, while juvenile fish one week old from hatching were no more tolerant to these wastes than were older fish.

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APPENDIX

TABLE 1.

Summarized Data from 24-hour Bioassays with Sticklebacks and Amphipods on Kraft Sample #10 Stored under Refrigeration at 1° to 2° C. in Closed Containers

Days of Storage	Sticklebacks		Amphipods	
	Non-aerated Bioassays	Aerated Bioassays	Non-aerated Bioassays	Aerated Bioassays
1st day	TL_m 1.7 %	TL_m 2.5 %	TL_m 3.25%	TL_m 4.95%
2*	2.0	2.5	4.65	5.7
5	2.35	4.78	3.0	7.42
8	3.72	>6.0	5.0	7.0
11	4.33	6.8	3.4	>10.0
18*	4.0	5.24	4.3	7.25

* Denotes liquor for bioassays taken from a full container. Liquor for other bioassays was taken from partially empty containers.

TABLE 2.

Summarized Data from 24-hour Bioassays with Sticklebacks and Amphipods on Kraft Sample #30 Stored at Room Temperatures of 18° ± 4° C. in Closed Containers

Days of Storage	Sticklebacks		Amphipods	
	Non-aerated Bioassays	Aerated Bioassays	Non-aerated Bioassays	Aerated Bioassays
1st day	TL_m 1.7 %	TL_m 2.5 %	TL_m 3.25%	TL_m 4.95%
3*	1.55	2.7	3.72	6.0
6	3.0	>6.0	4.76	6.7
9	3.0	>8.0	4.41	8.81
16*	2.5	5.0	5.0	6.28
23	3.8		4.4	

* Denotes liquor for bioassays taken from a full container. Liquor for other bioassays was taken from partially empty containers.

TABLE 3.

Summarized Data from 24-hour Bioassays with Sticklebacks and Amphipods on Kraft Sample #10 Stored at Room Temperatures of 18° ± 4° C. in Open Containers

Days of Storage	Sticklebacks		Amphipods	
	Non-aerated Bioassays	Aerated Bioassays	Non-aerated Bioassays	Aerated Bioassays
1st day	TL_m 1.7 %	TL_m 2.5 %	TL_m 3.25%	TL_m 4.95%
4*	2.17	2.83	4.1	5.25
7	>4.0	>6.0	7.0	>8.5
10	6.44	>10.0	<6.0	>10.0
17*	<4.0	<7.0	6.56	<9.0
24	5.5		7.4	

* Denotes liquor for bioassays taken from a full container. Liquor for other bioassays was taken from partially empty containers.

TABLE 4.

Summarized Data from 24-hour Bioassays with Sticklebacks and Amphipods on Kraft Sample #10 Stored Frozen in Paper Milk Cartons

Days of Storage	Sticklebacks		Amphipods	
	Non-aerated Bioassays	Aerated Bioassays	Non-aerated Bioassays	Aerated Bioassays
1st day	TL_m 1.7 %	TL_m 2.5%	TL_m 3.25%	TL_m 4.95%
19	> 5.0	> 6.0	> 6.0	> 9.0
25	5.6		> 6.0	

TABLE 5.

Summarized Data from 24-hour Bioassays with Sticklebacks and Amphipods on Kraft Sample #11 Stored in Full, Closed Containers at Different Temperatures

Refrigerated 1°- 2° C.		Room Temperatures 18° ± 4° C.		Outside Temperatures 2°- 15° C.		Frozen	
Days of Storage	24-hr. TL_m	Days of Storage	24-hr. TL_m	Days of Storage	24-hr. TL_m	Days of Storage	24-hr. TL_m
Sticklebacks							
1st day	2.2 %	1st day	2.2 %	1st day	2.2 %	1st day	2.2 %
5	3.15	6	2.29	7	3.57	2	4.0
12	2.0	13	2.9	14	2.86	8	7.0
19	3.08	20	2.59	21	2.88	15	6.0
40	2.0	41	2.0	42	2.62	48	6.33
Amphipods							
1st day	2.25%	1st day	2.25%	1st day	2.25%	1st day	2.25%
5	1.79	6	2.24	7	4.0	2	3.72
12	2.35	13	2.39	14	2.0	8	4.29
19	3.23	20	2.86	21	3.08	15	5.2
40	3.43	41	2.86	42	3.67	48	5.85

TABLE 6.

Comparison Between Bioassays Using Condensates Only and Those Using One Part Black Liquor to 99 Parts Condensates. The Threespine Stickleback was Used as the Bioassay Animal.

Date of Experiment	Kraft Sample Number	Liquor Tested	24-hr. $TL_{\underline{m}}$ as % by Volume of Liquor
6-21-58	23	Black Liquor and Condensates	3.27
6-21-58	23	Condensates only	3.83
7-20-58	24	Black Liquor and Condensates	3.38
7-20-58	24	Condensates only	3.27

TABLE 7.

Time of Death for Sticklebacks in Solutions with Different Proportions of Black Liquor, Recorded for 210 Minutes.

Testing Solution	Time of Death in Minutes for 20 Fish				
297 ml. condensates and	65	90	120	--	--
3 ml. black liquor (1:99)	120	120	--	--	--
per 2000 ml. solution	120	155	180	210	--
	120	180	--	--	--
297 ml. condensates and	120	155	155	180	210
6 ml. black liquor (2:99)	120	180	180	210	210
per 2000 ml. solution	120	155	180	180	180
	155	180	180	210	210
297 ml. condensates and	65	65	65	90	90
12 ml. black liquor (4:99)	65	65	65	120	120
per 2000 ml. solution	65	65	65	65	65
	65	65	90	120	155

TABLE 8.

Comparison of the Toxicity of 15 percent Kraft Liquors to Sticklebacks
Note: The total solids of black liquor were adjusted to the same total solids as 100 percent wash water, and duplicate bioassays were made with the pH adjusted to 7.4.

Liquor Tested	pH	Total Solids	Time of death in minutes for 10 fish					Average Time of Death
Condensates	7.4	trace	90 190	135 285	135 300	135 330	170 330	250.0 min.
Condensates	7.7	trace	150 230	150 230	190 230	190 230	230 385	221.5 min.
1:99*	7.4	0.15	45 60	45 85	45 85	55 85	55 85	64.5 min.
1:99*	8.7	0.15	40 80	40 80	50 80	50 80	50 105	65.5 min.
Black Liquor	7.4	0.2	430 1440	1200 1440	1200 --	1440 --	1440 --	--
Black Liquor	8.6	0.2	no mortalities in 24 hours					--
Wash Water	7.4	0.2	80 115	90 120	105 130	115 145	115 185	120.0 min.
Wash Water	7.9	0.2	430 1200	530 1200	1200 1440	1200 1440	1200 1440	1128.0 min.

* Wastes used were one part black liquor to 99 parts condensates.

TABLE 9.

Effects of pH on the Toxicity of Wash Water to Sticklebacks

Date of Experiment	Sample Number	pH of Testing Solution	24-hr. TL_m percent
7-11-58	2	9.0*	29.5
7-13-58	2	9.0*	27.7
7-27-58	3	9.3*	26.8
9-7-58	5	9.6*	30.0
9-20-58	6	9.3*	18.3
8-9-58	4	7.4	1.72
9-7-58	4	7.4	2.32
9-14-58	4	7.4	1.95
9-19-58	6	7.4	1.83

* The pH was estimated from testing dilutions used to determine the 24-hr. TL_m percent.

TABLE 10.

Effect of pH on the Toxicity of 5 percent Wash Water to Sticklebacks

Date	pH at Start	pH at 24 hours	Number of Fish	Fish Alive 24 hours	Percent Survival
8-24-58	4.0		5	0	0
8-24-58	5.0		5	2	40
8-24-58	6.0		5	0	0
8-24-58	7.0	no data	5	0	0
8-24-58	8.0		5	0	0
8-24-58	9.0		5	5	100
8-24-58	10.0		5	4	100
8-29-58	4.0		10	0	0
8-29-58	4.5		10	4	40
8-29-58	5.0		10	4	40
8-29-58	5.5	no data	10	0	0
8-29-58	6.0		10	0	0
8-29-58	6.5		10	0	0
8-29-58	7.0		10	0	0
8-30-58	7.5	7.3	10	0	0
8-30-58	8.0	7.5	10	0	0
8-30-58	8.5	8.0	10	10	100
8-30-58	9.0	8.55	10	10	100
8-30-58	9.5	9.1	10	10	100
8-30-58	10.0	9.4	10	10	100
8-30-58	10.4	9.8	10	10	100

TABLE 11.

The Tolerance of Sticklebacks of Various Mean Total Lengths to 5 percent Kraft Wastes

Size of Fish	Mean Total Length	Number of Fish	Survival 12 hours	Survival 24 hours
20-24 mm.	22.9 mm.	12	75 %	8.3%
25-29	25.5	10	80	60.0
30-34	31.9	13	100	54.0
35-39	36.0	4	100	50.0
40-44	41.5	16	93.7	87.5
45-49	46.6	12	100	58.0
50-54	52.3	6	100	66.0
55-59	56.7	4	100	75.0
60-65	62.3	3	100	0

TABLE 12.

Twenty-four-hour Bioassays on Eggs and Larval Sticklebacks Showing Tolerance with Age to Kraft Wastes at One Part Black Liquor to 99 Parts Condensates

Date of Experiment	Age* of Fish	Percent Concentration of Waste by Volume												24-hr. TL_m Percent
		Control	1.5	2	2.5	3	4	5	10	20	30	40	50	
		Percent Mortality												
8-10-58	0**	3			0			3	59	90				9.2
8-2-58	2½ hr.	5			10			30	40	85				12.2
7-25-58	5 days	0						0	20	72	100	100	100	15.8
8-9-58	6 days	0			13			0	65	40	100			8.6
8-3-58	7 days	0			5			0	95	100				7.5
8-3-58	8 days	0	0	0	0	0	0	0	80	80				8.2
8-3-58	8 days	0	0		0		0	0	80	75				8.2
8-17-58	9 days	0						50	100	100				5.0
8-3-58	14 days	0	5	10	10	20	90	100	100	100				3.5

* All ages were recorded from the time of fertilization.
** Eggs were fertilized immediately after they were placed in the waste solutions.