United States Department of the Interior, J. A. Krug, Secretary
Fish and Wildlife Service, Albert M. Day, Director

Special Scientific Report No. 37

STUDIES ON OIL EXTRACTS FROM *GRACILARIA CONFERVOIDES* (NORTH CAROLINA)

By

Charles F. Lee, Chemist, and Leonard S. Stoleff, former Chemist
Division of Commercial Fisheries

Washington
1948

Since long before the attack on Pearl Harbor there has been a widespread interest in sources of seaweed gums native to our own shores. Enemy occupation of vital points in the Pacific during the war cut off indefinitely the source of 92 percent of the prewar supply of agar, and threatened the small native source of supply from the exposed coast of California. These events led to an intensive search for substitutes which might replace agar in its various uses.

The industries and products in which agar plays a part are spread widely through our economy, and though its role may seem small, it is nevertheless an important cog in the works. To illustrate, a few uses are - as a conditioner in baked goods to prevent staling; as a jellying and surface improving agent for icings, pastry fillings, and candy jellies; as a stabilizing and bodying agent in pharmaceuticals, emulsions, ointments, and jellies; as a setting agent for dental impression materials; as a suspending agent for graphite in wire drawing lubricants; as a swelling agent in the coating of enteric pills; as a stabilizer and protective colloid in insecticidal sprays; as a humectant in tobacco mixtures; as a gelling agent for hectograph duplicators; as a gelling agent in the manufacture of leak-proof, non-spillable storage batteries; as a gelling agent and film former in the preparation of nitrogen fixing bacteria for addition to soils; for the preparation of sizes used in making photographic films; as a gelling agent for imbedding materials in the preparation of histological sections; as a gelling agent for bacteriological culture media. For many of the uses of agar, substitutes are readily available; for other uses, substitutes are not readily apparent. In any event, the loss of a major portion of our agar supply was a heavy drain on supplies of other jellying, emulsifying and stabilizing agents, themselves reduced in amount by curtailed imports and heavy wartime demands.

Among the seaweeds listed as being used for the extraction of agar was Gracilaria confervoides. Quantities of this weed have been reported as existing in South Africa, Australia, China, and Japan, and on the West Coast of the United States. Hoyt (1920) noted large quantities of this seaweed around Beaufort, North Carolina, and remarked upon the presence of a gel-forming component which was extractable with boiling water. Humm (1942) was, however, the first to thoroughly investigate the possibilities of this area. He estimated the amounts of seaweed to be of commercially exploitable magnitude, and reported his use of the gel-forming extract for bacteriological work. The possibility of an Atlantic agar industry based on these seaweed beds was suggested.

Following up Humm's findings, Stoloff (unpublished data) studied the extracts of several samples of Gracilaria confervoides from the North Carolina area. Methods of extraction and purification similar to those used in the preparation of agar yielded extracts which, in turn, produced gels of the same magnitude of strength as commercial agar gels of the same concentration. The Gracilaria extract gels, however, exhibited much greater syneresis and tended to set at a much higher temperature. This high setting temperature was found to be characteristic of one component which caused either complete gelation or thickening of the gel at high temperatures, depending on its concentration.

For many of the purposes for which agar has been used, as for example, in many of the food and pharmaceutical preparations, the physical properties mentioned have been of secondary importance. For bacteriological purposes, however, viscosity of the sol, gel strength, temperature of gelation, and amount of syneresis are determining factors in establishing the possibility of utilization of any seaweed extract.

It was for bacteriological use that a native agar, or agar substitute, was so critically needed. For this reason, when certain Gracilaria extracts were observed to contain little or none of the fraction gelling at a high temperature and to show only moderate syneresis, a more extensive investigation was outlined to determine seasonal variations in properties of the gum extracts.

Small scale commercial gathering of Gracilaria confervoides was started in 1943. Through the cooperation of three local fishermen it was possible to have weekly samples of seaweed gathered from three different locations, representative of different environments, and sent to the College Park, Maryland, laboratory of the Fish and Wildlife Service. Samples were collected weekly over the period from July 11 to December 30, 1944. During the early part of this period, both the attached "moplike" type of weed and the free floating "crinkly" variety were obtained from two of the beds.

The "crinkly" form of weed is thought to be entirely vegetative in reproduction, propagating from cells which have survived the winter in the bottom mud. Reproductive organs have never been observed on this type. The "moplike" form, on the contrary, almost always is covered with the nodules of these organs. The former type was the only one found after September 1. Storms made it impossible to collect samples for short periods, notably in August. During the last three months, when it was apparent that no more samples yielding gels of low gelling temperature were to be expected, only every second sample from each locality was extracted.

The samples, air-dried, contained considerable but varying amounts of moisture, free salt, sand, and debris, but no effort at purification prior to extraction was made. Two hundred to three hundred grams of the seaweed were added to 1½ to 2 gallons of water, in an open steam-jacketed copper kettle, and cooked at a slow boil for two hours. During the last half hour, several handfuls of "Hyflo Supercel" were added to assist filtration. The hot mixture was filtered through heavy duck, and the residue submitted to two additional extractions of about two hours each. The filtrates were allowed to gel in shallow pans and were then frozen. The blocks of frozen gel were placed on trays of 1/4 inch mesh screen, thawed and dried at room temperature with the aid of an electric fan. The resulting sheets of dried gum were ground, and the mixture was used for moisture determination and to prepare gels for physical tests.

This method of extraction and purification removed all except the very fine insoluble impurities. The salts and other soluble impurities remained in the gel, but most of these were removed in the water which separated on thawing the frozen gel. The remaining sheet of wet gum retained appreciable amounts of these soluble impurities, but was usually of a consistency which did not permit further washing. These electrolytes and soluble protein were known to have little effect upon the physical properties being studied, so no further refining was thought necessary.

Moisture was determined with the ground dried extract by heating a 2 to 3-gram sample in an air oven at 135° C. for three hours. Other data were determined with sols made up to a concentration of 1.5 percent dry weight of the extracted gum as calculated from data on the moisture content. Methods used in the determination of breaking load and syneresis are reported in more detail elsewhere. (Stoloff, 1944). Briefly, to determine the breaking load, the 1.5 percent sol was poured about 4 cm. deep in crystallizing dishes 7 cm. in diameter by 5 cm. high, allowed to set at room temperature for several hours, and then aged for 24 hours.

The force in grams required to rupture the gel surface (breaking load), when applied to a vertically held flat-ended plunger about one-half inch in diameter, was determined, and calculated as grams per centimeter of plunger circumference.

To determine water of syneresis, 100 ml. of the sol, cooled as near to the gel point as it could be poured, or about 50° to 55° C., were poured into a 250 ml. Erlenmeyer flask. The gel was allowed to set for one hour in a 20° C. incubator and then aged for at least 24 hours in a 37° C. incubator. The water of syneresis formed by this process was removed from the gel surface by repeated washings with carbon tetrachloride, and separated in a centrifuge tube with a graduated tip (A.S.T.M. oil tube) which permitted the amount of water to be read directly.

Stoloff (1944) had included a single sample of Gracilaria gum with a number of commercial agar samples in his study on syneresis, in which he concluded that this quantity approached a maximum after 24 hours. In the present and more extensive study of Gracilaria gels, however, it was observed that the amount of syneresis continued to increase for periods up to 72 hours. This observation was made accidentally after most of the gels had already been tested, using the 24 hour interval. This accounts for the considerably higher values noted for the most recently extracted gums and minimizes the significance of earlier values, particularly those reported as being under 1.5 ml. Two of the lowest values were checked over the 72 hour period and 3 to 5 times as much water of syneresis was obtained, but a general check of all these samples was not thought to be necessary, because the temperature of gelation alone makes most of these gum extracts unsuitable for general bacteriological use.

In this series of extracts, the exact temperature of gelation was not determined. The temperature of 45° C. was selected as a maximum at which a sol could set to a gel and still be used in pouring plates. Test tubes containing 10 ml. of the 1.5 percent sols were, therefore, held in a water bath at this temperature, and were rated simply as "plus" if the contents set to a gel, or "minus" if they remained liquid after one hour.

Most of those remaining liquid at 45° C. were tried out as media base and plated with samples of pasteurized milk. Four series were plated. In the first series, seven Gracilaria samples, and in the other series, these seven and two additional samples were plated in triplicate. The Tryptone-Glucose Extract media, recommended by the Standard Methods Committee of the American Public Health Association, was used in series 1 and 4, while series 2 and 3 were plated on a nutrient agar media, with the experimental Gracilaria gums being substituted, weight for weight, for the agar used in the control in every case. Series 3 was inoculated with 0.01 ml. of 4-day old milk, and the resulting counts were too small to be significant, nor did they indicate whether mixing had effectively distributed the inoculum. The same milk sample at 0.1 ml. in series 2 resulted in all plates being uncountable because of spreaders. Counts were obtained with all samples in series 1 and 4, using a 0.1 ml. inoculum of one day old milk, but many of these plates showed spreaders also, and only a few samples were consistently low in this respect (Table 1). Plates were all incubated at 37° C. for 48 hours.

Lumpiness of the sols, noted in more than half of the samples in series 3 and 4, in which the media were held at 45° C., was in part responsible for poor distribution of the inoculum in many of these plates. This temperature was so close to the gel point that the sol often set almost instaneously when coming in contact

with the glass of the Petri dish. In an effort to combat this effect, plates in series 1 were poured at 50° C., and even at this temperature good mixing was not obtainable. For proper distribution, a temperature of about 55° C. was judged necessary.

The higher temperature used in pouring plates with Gracilaria media, or possibly some difference in unknown nutritive factors in the presumably inert medium base, resulted in difference in counts on agar control and experimental plates. The counts using Gracilaria media were, in several cases, only 10 percent or less, of the controls. Similar differences were observed by Sandholzer (Stoloff, Lee, Sandholzer, et al, 1945) in plating several other typical Gracilaria extracts with a number of pure cultures. In the course of this investigation also, as in all previous work with Gracilaria, the high incidence of spreaders was noted. This undesirable property is apparently the result of the rather excessive amount of syneresis which these gels exhibit, as compared to agar. (Table 2).

Lumpiness before complete gelation was noted to some degree in almost all of the samples in the present series. This is apparently due to the presence of one or more components of the extract having high gelation temperatures, as observed by Stoloff (1943). There appears to be some of this high gelling temperature component in all of the samples, even though in some of the samples collected early in the season the amount is very small. Stoloff had attempted the separation of these gum fractions in 1943. This work met with little success, from which it is evident that the fractions are difficult to isolate, and there is little hope of a commercial process to economically eliminate the high gelling constituent.

This lumpiness and high viscosity above the temperature of complete gelation of some samples, and the high temperature of gelation of most samples, are, therefore, definite limiting factors in the use of any gums from North Carolina Gracilaria for bacteriological purposes. In addition, the excessive syneresis seems to be associated with the high incidence of spreaders and may cause gels to slip in the Petri dishes. Almost all of the samples gave irregularly lower plate counts as compared to an agar media with the same inoculum. All these factors are unfavorable to the general use of this gum for bacteriological work.

The data on percent yield and the three physical properties studied, are presented in Table 2. There is a large degree of variation present in all properties observed. Taking the group as a whole, the range in yields was from 19 to 43 percent, while for breaking load, an even greater range, from 60 to 340 grams per centimeter, was found. Syneresis showed the greatest variation, with values ranging from 0.6 to 5.0 milliliters. The corresponding coefficients of variation were found to be 18.9, 37.9, and 45.0 percent, respectively.

With this amount of variation it is not possible, with the small number of samples in each group, to show any relationship of breaking load, yield, or syneresis to source of sample, nor to type of sample, that is, crinkly, or noplike, attached weed. The only mean value that shows any indication of being a significant difference is that for the breaking load of the Oyster Farm samples, which is about 20 percent higher than the average breaking load of the other gel samples.

The relationship of the variables studied to date of collection is also not readily apparent. However, when all the values are plotted, as in Figures 1 to 3, it is possible to look beyond the numerous low and high values that unduly influence the statistical interpretation, and detect certain general trends. It is evident that there is no simple relationship continuing throughout the season for any of the three sets of data. Yield (Figure 1) shows less scatteration than the other data, and appears to increase to a maximum at about midsummer and then to decrease at a somewhat greater rate. In this case the data were divided, with September 23 included in series 1, and regression lines were calculated for each section. These are drawn in Figure 1, and the formulas are given in Table 3. For breaking load, there is an apparent rapid increase to a maximum in mid-August, an even more rapid decrease to an average value of about 160 grams per centimeter, and a further decline late in the season, after November 1. This is indicated by the broken line in Figure 2. The data for syneresis show what probably should be considered as entirely random distribution, with perhaps a slight increase in late season, aside from high values obtained with the increased aging period.

The date of collection does bear a positive relation to the temperature of gelation. Most of the sols which gelled below 45° C. were prepared from seaweeds collected before August 25. None was obtained from weed collected after September 25. The relation is not perfect, as some high gel-point seaweed was collected as early as August 4, but there seems little doubt that extracts with a temperature of gelation uniformly below 45° C. can only be expected from Gracilaria collected in the North Carolina area before August 1.

In summary, Gracilaria confervoides from Beaufort, North Carolina, was found to yield gum extracts amounting, on an average, to 26 to 23 percent of the dry weed. Gels of 1.5 percent (dry weight) concentration prepared from these extracts showed an average breaking load of about 165 grams per centimeter with a high degree of variability. The degree of syneresis exhibited by these gels, which averaged more than 2 milliliters per 100 grams of gel, makes them unsuited for routine bacteriological work. Certain of the extracts from seaweeds collected in July and early August gelled below 45° C., but the limited quantities of seaweed available at this period make commercial exploitation impractical. In several cases, with sols liquid at 45° C., high viscosity and lumpiness of the sol at temperatures 10° C., or more above gelation made proper mixing of media and inoculum difficult and limited their value for routine bacteriological work. The great majority of the extracted gums prepared in the course of this investigation set to solid or semi-solid gels at 45° C. in the concentration used, and possessed high viscosity amounting to a gluey consistency at quite high temperatures, rendering them unsuitable for bacteriological work involving a mixture of inoculum and liquid medium in the Petri dish.

Acknowledgment is made of the assistance of Dr. I. A. Sandholzer and Mrs. Margaret Dunn, bacteriologists of this Service, in connection with bacteriological studies reported for the Gracilaria extracts.

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TABLE 1. Plate Counts with Media Prepared with *Gracilaria* Extracts

Sample Number	Series 1		Series 4	
	Average Plate Count per milliliter	Spreaders, percent plates not countable**	Average Plate Count per milliliter+	Spreaders, percent plates not countable**
1	90	8	350	0
2	200	33	900	0
3	600	33	350	33
6	300	40	250*	0
8	80	16	700*	0
9	60	16	600	67
12	100	8	700*	33
15	-	-	600*	33
16	-	-	700*	33
Agar Control	620	0	3200	33

+ Count on plates showing growth.
* Agar contained lumps above temperature of gelation.
** Plates with more than 1/3 area covered by spreaders were not counted.

TABLE 2. Yield and Certain Physical Properties of Extracts from *Gracilaria confervoides* (North Carolina)

Source *	Type **	Sample Number ***	Collection date	Yield on dry basis	Breaking load	Syneresis, per 100 gm. gel	Gel at 45° C. + No gel at 45° C. -
				percent	grams per centimeter	milliliters	
OF	C	2	July 13	27	180	3.0	-
OF	C	6	July 21	29	80	1.8	-
BRP	C	9	July 26	38	170	1.4	-
EB	M	8	July 26	43	190	1.7	-
OF	C	10	July 28	22	190	1.7	-
OF	C	11	Aug. 4	30	260	1.5	+
BRP	M	12	Aug. 5	37	210	1.5	-
BRP	C	13	Aug. 5	30	230	2.3	+
EB	M	15	Aug. 10	30	230	2.9	-
BRP	C	16	Aug. 10	25	250	3.2	+
OF	C	14	Aug. 11	24	280	2.5	+
OF	C	17	Aug. 18	26	340	0.9	+
BRP	C	20	Aug. 19	25	220	1.2	+
BRP	M	21	Aug. 19	28	170	1.8	+
EB	C	18	Aug. 19	25	320	1.7	+
EB	M	19	Aug. 19	32	190	1.8	-
BRP	C	22	Aug. 25	25	100	0.6	-
BRP	M	24	Aug. 25	33	140	1.4	+
OF	C	23	Aug. 25	25	200	2.2	+
OF	C	25	Sept. 1	31	160	2.4	+
BRP	M	26	Sept. 2	28	140	1.6	-
BRP	C	27	Sept. 2	26.5	130	1.6	+
OF	C	29	Sept. 8	31	210	2.2	+
EB	C	32	Sept. 9	33	150	1.9	+
EB	C	30	Sept. 11	26.5	160	1.6	+
OF	C	31	Sept. 15	33	240	1.5	+
BRP	C	33	Sept. 17	35.5	150	1.5	+
OF	C	34	Sept. 21	40	160	2.0	+
BRP	C	35	Sept. 23	33	140	1.1	+
EB	C	41	Sept. 23	39	60	3.7	-
BRP	C	37	Sept. 28	22.5	180	1.4	+
OF	C	36	Sept. 29	32.5	150	1.5	+
OF	C	38	Oct. 6	28	200	2.2	+
BRP	C	40	Oct. 9	32	120	1.2	+
EB	C	43	Oct. 9	26.5	90	4.0	+
OF	C	42	Oct. 13	32	200	1.8	+
BRP	C	44	Oct. 23	27.5	180	2.4	+
EB	C	45	Oct. 23	27.5	190	1.7	+
OF	C	46	Oct. 27	27.5	120	1.2	+
BRP	C	48	Nov. 3	26.5	160	1.9	+
EB	C	49	Nov. 3	24	130	2.5	+
OF	C	47	Nov. 10	29	180	2.5	+
BRP	C	51	Nov. 20	19	100	3.8	+
EB	C	50	Nov. 20	24	140	4.0	+
OF	C	52	Nov. 24	20	120	4.1	+
BRP	C	54	Dec. 2	26	60	4.0	+
EB	C	53	Dec. 3	31	90	4.1	+
OF	C	55	Dec. 8	17	90	5.0	+
BRP	C	57	Dec. 16	20	70	3.3	+
EB	C	56	Dec. 16	26	90	2.7	+

* BRP-Bottle Rum Point. EB-Eastern Bay. OF-Oyster Farm.

** C-Free Floating Crinkly weed. M-Moplike attached weed.

*** Samples 1 and 3 were from seaweed collected on several different days and in different areas. Sample 4 was of *Hypnea musciformis*, and did not form a gel, and 5 contained some *H. musciformis* resulting in abnormal values for breaking load and syneresis. Sample 7 was from seaweed collected in January 1944.

TABLE 3. Summary of Data in Table 2, on Yield and Physical Properties of Extracts from *Gracilaria confervoides*.

Source of gums	Yield in dry weight		Breaking load per centimeter		Syneresis per 100 grams gel	
	Mean	Standard error	Mean	Standard error	Mean	Standard error
	percent	percent	grams	grams	milli-liters	milli-liters
Bottle Rum Point	28.3	1.23	154	11.90	1.96	0.220
Eastern Bay	29.8	1.62	156	19.46	2.64	0.278
Oyster Farm	28.0	1.25	187	15.59	2.22	0.236
Whole Group	28.58	0.764	166.2	8.88	2.23	0.142

Coefficient of variation for whole group. Yield, 18.9 percent; breaking load, 37.8 percent, and syneresis, 45.0 percent.

Regression line formula: For Yield.
July 12 to Sept. 23: Percent Yield = 28.0 + .0577 x (Number of days from 7/12).

Sept. 23 to Dec. 16: Percent Yield = 29.8 - .0875 x (Number of days from 9/23).

Correlation coefficients to Sept. 23: r = + 0.218
Sept. 24 to Dec. 16: r = - 0.524.

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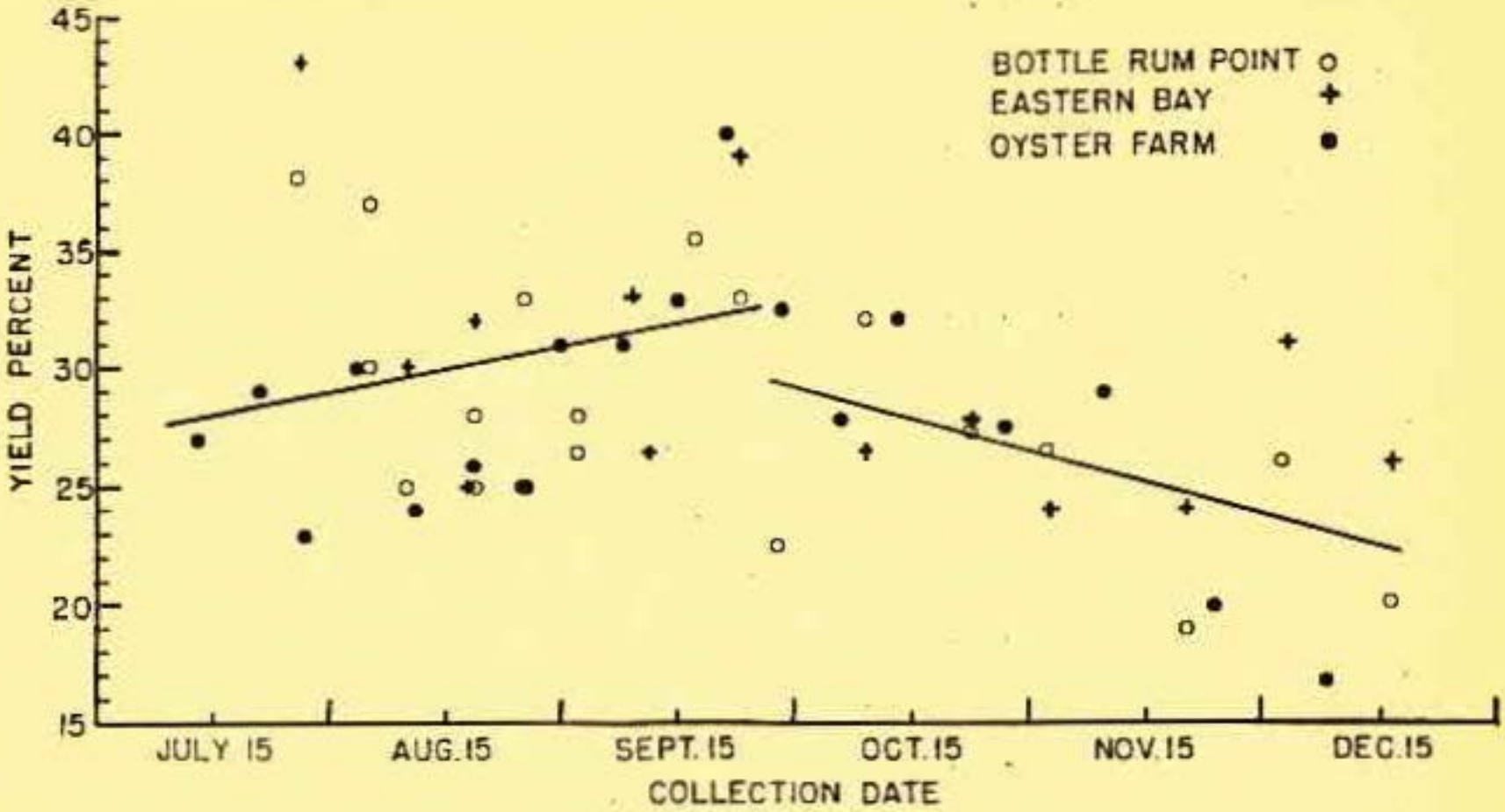

Figure 1. Percent yield, dry weight, of agar-like extract prepared from samples of *Gracilaria confervoides* collected near Beaufort, North Carolina, during 1944 season.

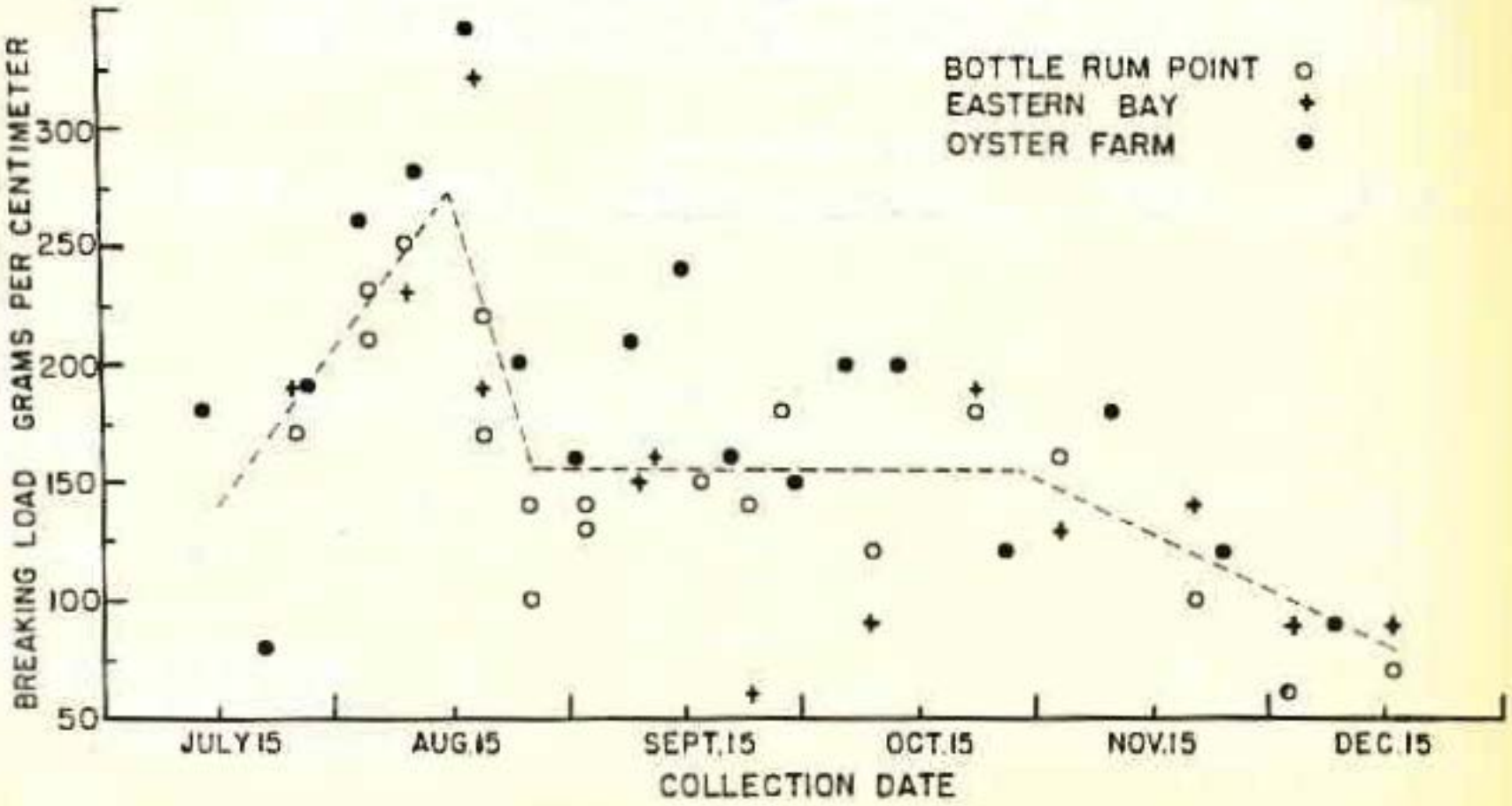

Figure 2. Breaking load of 1.5 percent gels from Gracilaria confervoides (North Carolina) showing relation of breaking load to source and date of collection, 1944 season.

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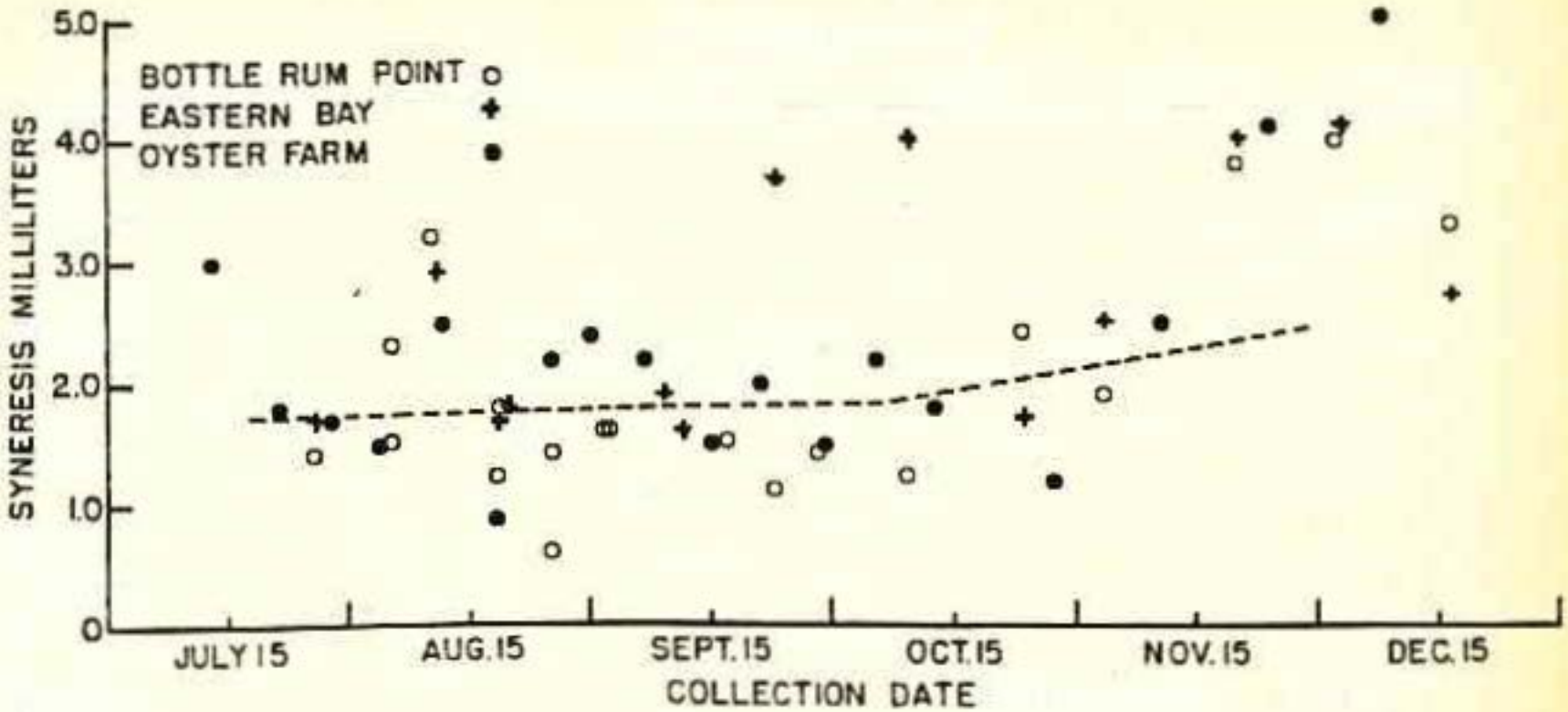

Figure 3. Syneresis of 1.5 percent gels from Gracilaria confervoides (North Carolina) showing relation of syneresis to source and date of collection, 1944 season. Determination on samples collected after November 15 were on gels aged 48 to 72 hours. Two other samples, collected September 23 and October 9, were checked with this procedure, resulting in the two high values in mid season.