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THE EFFECT OF GAMMA RAYS ON
MARINE FINFISH FILLETS INOCULATED WITH
TYPE E, CLOSTRIDIUM BOTULINUM

Final Report

[1968]

Massachusetts Institute of Technology
Cambridge, Massachusetts

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THE EFFECT OF GAMMA RAYS ON MARINE FINFISH FILLETS INOCULATED WITH
TYPE E, CLOSTRIDIUM BOTULINUM

Final Report

July 15, 1967 - July 14, 1968

S.A. Goldblith and J.T.R. Nickerson

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Division of Isotopes Development
United States Atomic Energy Commission
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Report 1. DETERMINATION OF SPOILAGE TIMES OF VACUUM PACKED,
IRRADIATED AND NONIRRADIATED HADDOCK STORED AT 38,42,
45 AND 50°F. SPOILAGE DETERMINED BY ODOR.

SUMMARY

The total rejection time for vacuum-packed nonirradiated and irradiated haddock was determined for storage temperatures of 38, 42, 45 and 50°F. A summary of the results of such tests is given in the following table:

Irrad. Dose (Krad)	Storage Temp. (°F)	Approximate	Vacuum Packed Storage Life (days)
		Air Packed Storage Life* B.C.F. date (days)	
none	38	14	29
	42	11	28
	45	10	18
	50	7	14
100	38	24	56
	42	22	35
	45	15	29
	50	12	36
200	38	35	>112
	42	30	>84
	45	19	84
	50	18	84

*Data supplied by Mr. Louis Ronsivalli - Bureau of Commercial Fisheries

The rejection time for vacuum-packed haddock fillets has been determined as that period in which all six testers considered the sample to be spoiled, judging on the basis of odor alone. This is the method used by the Bureau of Commercial Fisheries to determine storage life time of X values. Regarding the growth of *Clostridium botulinum*, type E, in inoculated packs, rejection of samples on the basis of odor alone is the most rigorous criterion which could be used since many of the samples may have been rejected at earlier

periods on the basis of appearance.

METHODS

Preparation of Samples

Discs of flesh, the thickness of the fillet, weighing approximately 100 g., were cut out of haddock fillets and placed in metal cans (307 x 113). The cans were sealed under a vacuum of 25 to 26 inches (Hg) in the container. Aerobic control samples were not prepared.

The cans containing the samples were either not irradiated or were irradiated at 100 or 200 krad and were then stored at 50, 45, 38°F.

Testing for Spoilage

After definite storage periods the cans were removed from storage, opened and six persons were required to smell the samples. When all testers considered the samples to be spoiled according to odor, this period was considered to be the spoilage time. This method of determining spoilage times was used since the Bureau of Commercial Fisheries has determined storage life times in this manner.

Bacterial Counts

At specified times a portion of 50 g. of haddock flesh was added to 450 ml. of sterile phosphate buffer (40°F) in a sterile blender top and blended for 3 minutes. Serial dilutions were made from this and were surface plated in pre-poured Eugon plus 1 per cent yeast extract agar (15 g. trypticase, 10 g. of yeast extract, 5 g. phytone, 4 g. sodium chloride, 0.2 g. sodium sulfite, 0.7 g. l-cystine, 5.5 g. dextrose and 15 g. agar, in one liter of distilled water). The plates

were incubated aerobically at 68°F for 5 days prior to counting.

Anaerobic cultures were prepared by surface plating on pre-poured APT agar plates (tryptone 12.5 g., dextrose 10.0 g., yeast extract 7.5 g., sodium citrate 5.0 g., sodium chloride 5.0 g., dipotassium phosphate 5.0 g., magnesium sulfate 0.8 g., manganese chloride 0.4 g., sorbitan monooleate complex 0.2 g., ferrous sulfate 0.04 g., thiamine hydrochloride 0.0001 g., agar 15 g., in one liter of water). These cultures were incubated in air tight anaerobic jars in which BBL-H₂-CO₂ gas packs has been added to remove oxygen and provide the right concentration of carbon dioxide. Counts were made after incubating for 5 days at 68°F. A methylene blue test paper was used in the container to determine that anaerobiosis had been maintained.

DISCUSSION OF RESULTS

The results of 100 per cent spoilage time tests with aerobic and anaerobic bacterial counts are shown in Table 1. A table of spoilage times for both aerobic and anaerobic packs has also been given in the summary. It can be seen that, excepting for the 38 and 42°F, 100 krad treated samples, spoilage times for the anaerobic pack, not irradiated or irradiated at 100 krad, are usually at least twice as long as that for the aerobic pack and that for products irradiated at 200 krad the spoilage time for the anaerobic pack is much more than twice as long as that of the aerobic pack. Spoilage times for the 38 and 42°F, 100 krad treated samples, were approxi-

mately 1 1/2 as long as that for the aerobic pack. It should be noted, however, that anaerobic packs might have been rejected on the basis of appearance long before this was done on the basis of odor alone, had the former been considered as a criterion of spoilage during the tests. This may also be true of aerobic packs.

Since an additional pack of fish was required for the detection of spoilage of some of the 200 krad irradiated vacuum packed samples, these results are given in Table 2. It will be noted that spoilage times for vacuum packed, 200 krad irradiated samples stored at 42 or at 38°F, were not determined since these times, according to odor tests, are longer than 84 and 112 days, respectively.

At the time of spoilage, as determined by odor test, both the aerobic and anaerobic bacterial counts on vacuum packed samples were in the 10^7 and 10^8 per gram range. Aerobic counts did not differ greatly from anaerobic counts at the time of spoilage, indicating that the spoilage bacteria were facultative types.

TABLE 1. Bacterial Plate Counts and Odor Test Reactions for
Skinless Haddock Fillets Packed Under Vacuum and
Irradiated at 0, 100 and 200 krad and Stored at
50°, 45°, and 42° and 38°F.

Storage Temperature (°F)	Radiation Dose (krad)	Storage Time (days)	Anaerobic Count (Bacteria/gram)		Aerobic Count (Bacteria/gram)		Rejection %
			Sample 1	Sample 2	Sample 1	Sample 2	
50	0	4	5.6x10 ⁵	9.0x10 ⁵	1.1x10 ⁶	1.3x10 ⁶	
		8	1.0x10 ⁷	1.4x10 ⁷	2.0x10 ⁷	1.9x10 ⁷	
		14	2.1x10 ⁷	7.1x10 ⁷	2.9x10 ⁷	8.1x10 ⁷	100
	100	7	1.4x10 ⁷	1.0x10 ⁷	1.6x10 ⁷	1.0x10 ⁸	
		11	4.2x10 ⁷	9.5x10 ⁸	3.5x10 ⁸	9.1x10 ⁸	
		17	2.6x10 ⁸	2.9x10 ⁸	1.9x10 ⁸	3.0x10 ⁸	
		22	2.3x10 ⁸	3.0x10 ⁸	3.1x10 ⁸	2.0x10 ⁸	
		28	2.6x10 ⁸	1.1x10 ⁸	1.6x10 ⁸	1.6x10 ⁸	
		32	1.7x10 ⁸	1.4x10 ⁸	3.1x10 ⁸	2.2x10 ⁸	
		36	2.0x10 ⁸	2.9x10 ⁸	2.8x10 ⁸	2.0x10 ⁸	100
	200	7	2.2x10 ⁴	3.7x10 ⁴	2.1x10 ⁴	3.3x10 ⁴	
		14	1.7x10 ⁵	3.4x10 ⁵	1.5x10 ⁵	3.9x10 ⁵	
		21	4.0x10 ⁷	8.5x10 ⁸	3.6x10 ⁸	8.6x10 ⁸	
		28	1.7x10 ⁸	1.8x10 ⁷	2.3x10 ⁸	1.0x10 ⁸	
		35	8.4x10 ⁷	6.0x10 ⁷	1.4x10 ⁸	5.6x10 ⁸	
		39	2.2x10 ⁸	1.2x10 ⁷	1.3x10 ⁸	2.7x10 ⁷	
		45	2.6x10 ⁸		2.5x10 ⁸		
45	0	4	1.5x10 ⁵	2.4x10 ⁵	2.2x10 ⁵	5.5x10 ⁵	
		8	1.1x10 ⁶	4.7x10 ⁶	1.2x10 ⁶	4.1x10 ⁶	
		14	3.2x10 ⁶	8.0x10 ⁶	6.0x10 ⁶	9.4x10 ⁶	
		18	1.2x10 ⁷	1.6x10 ⁸	1.4x10 ⁷	2.1x10 ⁸	100
	100	6	3.6x10 ⁴	8.9x10 ⁵	3.3x10 ⁴	9.0x10 ⁵	
		13	3.8x10 ⁷	9.4x10 ⁷	4.4x10 ⁶	14.x10 ⁸	
		17	5.5x10 ⁶	2.3x10 ⁷	8.5x10 ⁷	4.0x10 ⁷	
		23	9.5x10 ⁷	1.0x10 ⁸	8.4x10 ⁸	9.8x10 ⁷	
		29	1.2x10 ⁸	4.4x10 ⁸	1.1x10 ⁸	2.8x10 ⁸	100
	200	7	4.0x10 ³	6.0x10 ³	5.0x10 ³	5.7x10 ³	
		14	2.5x10 ³	4.2x10 ³	2.1x10 ³	4.6x10 ³	
		21	2.6x10 ⁶	2.4x10 ⁶	3.1x10 ⁶	1.4x10 ⁷	
		28	8.9x10 ⁷	4.6x10 ⁷	8.4x10 ⁷	4.9x10 ⁷	
		35	7.5x10 ⁷	1.6x10 ⁸	5.6x10 ⁷	1.5x10 ⁸	
		42	4.2x10 ⁷	2.4x10 ⁷	6.4x10 ⁷	2.8x10 ⁷	
		49	2.6x10 ⁸	1.6x10 ⁸	1.2x10 ⁸	1.8x10 ⁸	
		56	1.4x10 ⁸	1.1x10 ⁸	1.7x10 ⁸	1.5x10 ⁸	<100

TABLE 2. Bacterial Counts and Odor Test Reactions for
Skinless Haddock Fillets Packed under Vacuum
Irradiated at 200 krad and Stored at 42, 45,
and 50°F.

Storage Temperature (°F)	Radiation Dose (krad)	Storage Time (days)	Anaerobic Count (Bacteria/gram)		Aerobic Count (Bacteria/gram)		Rejection %
			Sample 1	Sample 2	Sample 1	Sample 2	
50	200	49	1.2x10 ⁸	9.4x10 ⁷	6.7x10 ⁷	7.9x10 ⁷	100
		56	1.2x10 ⁸	1.1x10 ⁸	9.6x10 ⁷	9.9x10 ⁷	
		63	9.6x10 ⁷	6.2x10 ⁷	7.2x10 ⁷	7.3x10 ⁷	
		70	7.1x10 ⁸	8.8x10 ⁷	8.6x10 ⁸	9.8x10 ⁷	
		77	1.4x10 ⁸	9.4x10 ⁸	1.1x10 ⁸	9.8x10 ⁸	
		84	1.8x10 ⁸	2.1x10 ⁸	2.0x10 ⁸	1.8x10 ⁸	
45	200	49	1.3x10 ⁸	9.4x10 ⁷	1.2x10 ⁸	7.6x10 ⁷	100
		56	7.4x10 ⁷	6.3x10 ⁷	6.8x10 ⁷	5.5x10 ⁷	
		63	4.9x10 ⁷	5.0x10 ⁷	4.8x10 ⁷	4.7x10 ⁷	
		70	3.4x10 ⁷	6.4x10 ⁷	7.0x10 ⁷	7.3x10 ⁷	
		77	5.5x10 ⁷	1.4x10 ⁷	1.9x10 ⁷	1.5x10 ⁷	
		84	4.6x10 ⁷	5.5x10 ⁷	5.0x10 ⁷	5.0x10 ⁷	
42	200	49	6.9x10 ⁷	9.2x10 ⁷	4.6x10 ⁷	8.3x10 ⁷	<100
		56	1.2x10 ⁸	6.6x10 ⁷	1.1x10 ⁸	8.8x10 ⁷	
		63	6.8x10 ⁷	8.0x10 ⁸	6.8x10 ⁷	9.4x10 ⁸	
		70	2.0x10 ⁷	1.7x10 ⁷	4.5x10 ⁷	1.9x10 ⁷	
		77	7.0x10 ⁷	7.2x10 ⁷	5.8x10 ⁷	5.3x10 ⁷	
		84	2.9x10 ⁷	4.3x10 ⁷	4.2x10 ⁷	5.7x10 ⁷	

Report 11 TOXICITY OF VACUUM-PACKED HADDOCK, INOCULATED
WITH TYPE E CL. BOTULINUM, IRRADIATED AND NON-
IRRADIATED AND HELD AT VARIOUS TEMPERATURES.

SUMMARY

In studies to determine the toxicity of haddock inoculated with type E *Cl. botulinum*, vacuum-packed, irradiated or not irradiated and stored at 50, 45, 42 or 38°F, various results were obtained depending upon the treatment applied. A summary of these results is shown in Table 3.

Table 3.
Toxicity of Inoculated Haddock Samples

Approximate Inoculum Level (spores/g)	Dose Applied (krad)	Storage Temperature (degrees F)	Rejection Time by Odor (days)	Time at which Samples were Toxic (days)
10^6	0	50	14	7
	100	50	36	28
	200	50	84	35
10^4	0	50	14	7
	100	50	36	28
	200	50	84	35
10^2	0	50	14	7
	100	50	36	28
	200	50	84	35
10^6	0	45	18	18
	100	45	29	56
	200	45	84	28
10^4	0	45	18	27
	100	45	29	28
	200	45	84	28
10^2	0	45	18	>38
	100	45	29	56
	200	45	84	56

Approximate Inoculum Level (spores/g)	Dose Applied (krad)	Storage Temperature (degrees F)	Rejection Time by Odor (days)	Time at which Samples were Toxic (days)
10^6	0	42	28	26
	100	42	35	>70
	200	42	>84	>94
10^4	0	42	28	39
	100	42	35	>70
	200	42	>84	126
10^2	0	42	28	26
	100	42	35	>70
	200	42	>84	63
10^6	0	38	29	>50
	100	38	56	>112
	200	38	>112	>112
10^4	0	38	29	>50
	100	38	56	>112
	200	38	>112	112
10^2	0	38	29	50
	100	38	56	112
	200	38	>112	>112

At 50°F all samples, including the controls, 100 krad irradiated and 200 krad samples became toxic prior to the total rejection time for vacuum-packed samples.

At 45°F the controls and the 100 krad samples became toxic at rejection time for vacuum-packed samples or at periods somewhat longer than that of rejection time. At 45°F the 200 krad irradiated samples became toxic prior to the rejection time for vacuum-packed samples.

At 42°F the controls became toxic at the rejection time for vacuum-packed samples or at periods somewhat longer

than this. The 100 krad irradiated samples held at 42°F did not become toxic after periods of at least twice that of total rejection for vacuum-packed samples treated at this dose level. Some of the samples irradiated at 200 krad became toxic prior to the total rejection time for vacuum-packed samples treated at this level.

At 38°F the controls and the 100 krad treated samples became toxic or did not become toxic after periods considerably greater than the total rejection time for vacuum-packed controls or for vacuum-packed 100 krad treated samples, respectively. Some of the 200 krad treated samples became toxic at 38°F prior to the total rejection time for vacuum-packed samples treated at this level.

METHODS

Production and Preparation of Spores of type E *Cl. botulinum*.

The Beluga strain of type E *Cl. botulinum* was inoculated into 10 ml portions at deaerated trypticase-peptone-glucose (TPG-t) broth containing 0.2 per cent sodium thioglycollate and heat shocked at 140°F for 15 minutes. The broth was then stratified with vaspar and incubated at 86°F for 18 to 24 hours. The incubated tubes of broth were then each transferred to 90 ml. of deaerated TPG-t broth, the new cultures were stratified with vaspar and incubated at 86°F until about 80 per cent of spore formation had occurred (approximately 36 hours). These cultures were then held at 45°F for 18 to 20 hours prior to centrifuging to harvest the spores.

The cultures were centrifuged at 8000 RPM in a refrigerated centrifuge for 20 minutes and the supernatant discarded. The spores were then resuspended in distilled water and recentrifuged. This was repeated twice. After the final centrifugation the sediment containing the spores was suspended in 20 ml of distilled water and held at 33°F until used for inoculation of fish tissues. Prior to using for inoculation, the spore suspension was counted using Eugon plus yeast extract agar to determine the number of spores or spores and cells per milliliter.

Preparation of Inoculated Fish Packs.

Skinless haddock fillets purchased from a local filleting plant were cut into 50 grams discs and placed in C-enamel

lined, 307x113, metal cans. Each fish portion was inoculated with one ml. of heat-shocked spore suspension (heated at 140°F for 15 minutes) or 1 ml. of unheat-shocked spores or spores and cells, a number of areas of each portion being injected. The spore or spores and cell concentration of each ml of suspension used for injection was such that each fish portion received approximately 1×10^2 , 1×10^4 or 1×10^6 spores or spores and cells per gram. The cans were then sealed under vacuum to provide a 25 to 26 inch vacuum in the container. Samples prepared in this manner were either not irradiated or were irradiated at 100 or 200 krad followed by storage at 50, 45, or 38°F until tested for toxin after one-half, one, one and one-half or two times the storage life (as determined by odor) for the product subjected to the particular treatment.

Bacterial Counts and Toxicity Tests on Inoculated Packs.

Each of two 50 gram portions were removed from the cans blended with 100 ml of gel-phosphate buffer (pH 6.2). A portion of 10 ml of the blend was then transferred to 33 ml of refrigerated phosphate buffer (pH 7.0) to provide a 1:10 dilution to be used for bacterial counts. The remainder of the 1:2 blend was held overnight at 33°F prior to testing for toxin. The bacterial count suspension was further diluted serially; then cultures were prepared in Miller-Prickett tubes using TPG agar containing 0.05 per cent sodium thioglycollate, 0.01 per cent sodium bicarbonate, 0.05 per cent ferric citrate and 0.07 per cent sodium sulfite. Cultures were

incubated at 86°F. Counts were made after 18 to 36 hours, clostridia being counted as black colonies. Aerobic counts were made by surface plating on Eugon agar containing 0.5 per cent yeast extract and incubating cultures at 68°F for 5 days prior to making counts.

For toxin assays the 1:2 blend of fish and gel-phosphate buffer held overnight at 33°F was centrifuged under refrigeration at 8000 RPM for 20 minutes. A portion of the supernatant was trypsinized (1 per cent of trypsin added) at 98.6°F for 45 minutes after which portions of 0.5 ml of both trypsinized and nontrypsinized extracts were injected intraperitoneally into one protected (type E *Cl. botulinum* antitoxin) and two unprotected white male mice. The mice were then observed over a period of 72 hours for symptoms of botulism and death.

DISCUSSION OF RESULTS

The results of bacterial counts and toxicity tests on haddock, inoculated with spores of type E *Cl. botulinum*, irradiated or not irradiated and held at 50, 45, 42 and 38°F are shown in tables 4, 5, 6, and 7. A summary of the results is given in table 3.

The results of toxicity tests as summarized in table 3 can be interpreted to mean that the use of 200 krad to treat haddock imposes a public health hazard since vacuum-packed type E *Cl. botulinum* inoculated samples treated at this level were more often toxic prior to the end of the spoilage period for such samples than were the nonirradiated samples at the

time of spoilage. Since the opposite was the case with the 100 krad treated samples (unirradiated controls more often toxic at the time of spoilage than were the 100 krad treated samples at the time of spoilage), if treatment at 200 krad is to be considered to impose a public health hazard, treatment at 100 krad must be considered to provide a public health safety factor.

It is not believed that irradiation treatment of 200 krad caused changes in the composition of haddock which favored the growth of type E *Cl. botulinum*. The reason why the 200 krad treated samples were more often toxic would seem to have been caused by the extremely long spoilage times provided by the combination of vacuum packing and treatment at a level of 200 krad. Whereas, therefore, in most cases with vacuum-packed controls and with the vacuum-packed 100 krad treated samples, the product would be spoiled prior to outgrowth and toxin production by type E *Cl. botulinum*; in the case of the vacuum-packed 200 krad treated samples the spoilage times were long enough to allow the outgrowth of the organism with toxin production.

TABLE 4. Bacterial Counts and Toxicity Data for Skinless Haddock Fillets Inoculated with *Cl. botulinum*, type E (Beluga), Sealed under Vacuum, Irradiated and Stored at 50°F.

Inoculum Level (Spores/gm)	Radiation Dose (krad)	Storage Time (days)	Clostridial Count (Bacteria/gram)		Facultative Count (Bacteria/gram)		Toxicity	
			Sample 1	Sample 2	Sample 1	Sample 2	1	2
10 ⁶	0	7	>1.0x10 ⁵	>1.0x10 ⁵	6.5x10 ⁷	5.5x10 ⁷	+	+
		14	7.0x10 ⁵	1.2x10 ⁵	1.8x10 ⁸	1.8x10 ⁸	+	+
10 ⁴		7	1.5x10 ⁴	>5.0x10 ⁴	1.6x10 ⁸	5.0x10 ⁷	+	+
		14	7.0x10 ⁵	2.0x10 ⁶	1.0x10 ⁸	9.1x10 ⁷	+	+
10 ²		7	>5.0x10 ⁴	<10 ¹	1.1x10 ⁸	1.0x10 ⁸	+	+
		14	7.0x10 ⁵	4.3x10 ⁵	2.8x10 ⁸	2.0x10 ⁸	+	+
10 ⁶	100	14	<10 ¹	<10 ¹	3.9x10 ⁷	1.4x10 ⁸	-	-
		28	6.0x10 ⁵	5.1x10 ⁵	6.0x10 ⁷	3.8x10 ⁷	+	+
10 ⁴		14	<10 ¹	LOST	3.2x10 ⁸	LOST	-	LOST
		28	>1.0x10 ⁵	>1.0x10 ⁵	5.1x10 ⁷	6.7x10 ⁷	+	+
10 ²		14	<10 ¹	<10 ¹	3.4x10 ⁸	4.4x10 ⁸	-	-
		28	>1.0x10 ⁵	>1.0x10 ⁵	3.0x10 ⁸	6.0x10 ⁸	+	+
10 ⁶	200	35	4.8x10 ⁶	<10 ²	4.8x10 ⁷	6.8x10 ⁷	+	+
10 ⁴		35	5.5x10 ⁶	4.5x10 ²	2.5x10 ⁷	4.0x10 ⁸	+	+
10 ²		35	>1.0x10 ⁶	9.0x10 ²	3.8x10 ⁷	1.3x10 ⁷	+	+

TABLE 5. Bacterial Counts and Toxicity Data for Skinless Haddock Fillets Inoculated with *Cl. botulinum*, type E (Beluga), Sealed under Vacuum, Irradiated and Stored at 45°F.

Inoculum Level (Spores/gm)	Radiation Dose (krad)	Storage Time (days)	Clostridial Count (Bacteria/gram)		Facultative Count (Bacteria/gram)		Toxicity	
			Sample 1	Sample 2	Sample 1	Sample 2	1	2
10^6	0	8	1.0×10^3	5.6×10^3	8.6×10^6	9.8×10^6	-	-
		18	8.0×10^4	3.6×10^5	3.5×10^7	4.8×10^7	-	+
		27	1.5×10^6	3.2×10^6	1.1×10^8	9.6×10^7	+	+
10^4		8	$<10^1$	$<10^1$	1.3×10^7	9.2×10^6	-	-
		18	$<10^1$	3.0×10^3	1.3×10^8	7.8×10^7	-	-
		27	4.1×10^5	3.2×10^5	8.3×10^7	5.8×10^7	+	+
10^2		8	$<10^1$	$<10^1$	1.2×10^7	6.4×10^7	-	-
		18	$<10^1$	$<10^1$	3.7×10^7	2.7×10^7	-	-
		27	8.0×10^3	2.0×10^3	9.0×10^8	5.5×10^7	-	-
		38	1.4×10^4		1.1×10^8		-	-
10^6	100	14	$<10^1$	$<10^1$	5.7×10^7	3.6×10^7	-	-
		28	$<10^1$	$<10^1$	6.4×10^8	6.9×10^8	-	-
		42	$<10^1$	0.5×10^4	2.8×10^8	1.5×10^8	-	-
		56	$>1.0 \times 10^4$	$>1.0 \times 10^4$	1.1×10^8	1.0×10^8	+	+
10^4		14	$<10^1$	$<10^1$	4.4×10^8	3.1×10^8	-	-
		28	2.0×10^2	1.0×10^3	1.4×10^8	6.2×10^7	+	-
		42	3.3×10^5	1.5×10^4	3.5×10^7	3.0×10^8	+	+
		56	1.0×10^3	1.0×10^3	2.3×10^8	2.9×10^8	+	+
10^2	100	14	LOST	$<10^1$	LOST	3.8×10^7	LOST	-
		28	$<10^1$	$<10^1$	7.5×10^7	1.5×10^8	-	-
		42	$<10^1$	$<10^1$	4.6×10^7	1.0×10^8	-	-
		56	$<10^1$	$<10^1$	6.3×10^8	1.1×10^8	-	+
10^6	200	28	1.9×10^5	3.1×10^2	5.0×10^4	1.7×10^7	+	+
10^4		28	gas at 10^{-3} $<10^1$ black col.	gas at 10^{-3} col.	1.4×10^6	1.0×10^5	+	+
10^2		28	$<10^1$	$<10^1$	1.2×10^8	1.4×10^7	-	-
		56	2.0×10^4	5.0×10^4	4.0×10^7	4.2×10^7	+	+

TABLE 6. Bacterial Counts and Toxicity Data for Skinless Haddock Fillets Inoculated with *Cl. botulinum*, type E (Beluga), Sealed under Vacuum, Irradiated and Stored at 42°F.

Inoculum Level (Spores/gm)	Radiation Dose (krad)	Storage Time (days)	Clostridial Count (Bacteria/gram)		Facultative Count (Bacteria/gram)		Toxicity	
			Sample 1	Sample 2	Sample 1	Sample 2	1	2
10^6	0	13	1.5×10^3	1.2×10^3	9.0×10^6	4.8×10^6	-	-
		26	1.2×10^3	6.0×10^3	5.9×10^7	5.0×10^8	+	-
		39	3.0×10^3	3.3×10^3	9.4×10^7	1.0×10^8	+	+
		50	2.0×10^3	2.0×10^3	6.0×10^7	1.5×10^8	+	-
10^4		13	0.5×10^1	$<10^1$	6.9×10^6	3.8×10^6	-	-
		26	gas ₁ at 10^{-3} $<10^1$ black	4.0×10^2	1.0×10^7	5.0×10^7	-	-
		39	col. gas ₁ at 10^{-3} $<10^1$ black	gas ₁ at 10^{-3} $<10^1$ black	6.2×10^7	5.8×10^7	-	-
		50	col. 3.5×10^3	col. 2.0×10^3	1.3×10^8	6.6×10^7	+	+
10^2		13	$<10^1$	$<10^1$	3.8×10^6	4.0×10^6	-	-
		26	gas ₁ at 10^2 $<10^1$ black	gas ₁ at 10^2 $<10^1$ black	5.1×10^7	6.4×10^7	-	+
		39	col. gas and blackening at 10^2	col. gas and blackening at 10^2	1.7×10^8	1.1×10^8	-	-
		50	$<10^1$	$<10^1$	4.8×10^8	1.2×10^0	-	-
10^6	100	18	0.5×10^1	0.5×10^1	3.6×10^7	7.2×10^7	-	-
		35	$<10^1$	$<10^1$	5.2×10^8	3.8×10^7	-	-
		53	$<10^1$	$<10^1$	1.3×10^7	7.7×10^5	-	-
		70	$<10^1$	$<10^1$	3.9×10^7	6.0×10^5	-	-
10^4		18	$<10^1$	$<10^1$	2.4×10^7	6.1×10^8	-	-
		35	$<10^1$	$<10^1$	4.0×10^6	6.0×10^7	-	-
		53	$<10^1$	$<10^1$	4.8×10^8	3.7×10^7	-	-
		70	$<10^1$	$<10^1$	1.3×10^8	5.1×10^7	-	-
10^2		18	$<10^1$	$<10^1$	4.6×10^8	5.1×10^7	-	-
		35	$<10^1$	$<10^1$	1.9×10^8	1.7×10^8	-	-
		53	$<10^1$	$<10^1$	3.4×10^7	1.1×10^8	-	-
		70	$<10^1$	$<10^1$	1.5×10^8	6.0×10^7	-	-
10^6	200	35	$<10^1$	$<10^1$	9.7×10^6	7.3×10^7	-	-
		63	$<10^1$	$<10^1$	contamina-	1.5×10^4	-	-
		95	$<10^1$	$<10^1$	tion 5.4×10^7	1.0×10^8	-	-
		126	$<10^1$	$<10^1$	1.6×10^8	1.8×10^8	-	-

TABLE 6.

Inoculum Level (Spores/gm)	Radiation Dose (krad)	Storage Time (days)	Clostridial Count (Bacteria/gm)		Facultative Count (Bacteria/gram)		Toxicity	
			Sample 1	Sample 2	Sample 1	Sample 2	1	2
10^4	200	35	10^1	10^1	1.8×10^7	1.3×10^6	-	-
		63	10^1	10^1	2.0×10^8	2.3×10^7	-	-
		95	10^1	10^1	1.0×10^8	6.7×10^8	-	-
		126	10^1	10^1	1.6×10^8	1.2×10^8	-	-
10^2		35	10^1	10^1	1.7×10^8	9.7×10^6	-	-
		63	10^1	1.2×10^5	1.0×10^8	5.0×10^5	-	+
		95	1.2×10^4	2.2×10^4	1.2×10^8	8.3×10^7	+	+
		126	10^1	10^1	1.8×10^8	1.5×10^7	-	-

TABLE 7. Bacterial Counts and Toxicity Data for Skinless Haddock Fillets Inoculated with *Cl. botulinum*, type E (Beluga), Sealed under Vacuum, Irradiated and Stored at 38°F.

Inoculum Level (Spores/gm)	Radiation Dose (krad)	Storage Time (days)	Clostridial Count (Bacteria/gram)		Facultative Count (Bacteria/gram)		Toxicity	
			Sample 1	Sample 2	Sample 1	Sample 2	1	2
10^6	0	13	1.2×10^3	1.0×10^3	7.4×10^6	5.4×10^6	-	-
		26	$<10^1$	$<10^1$	3.1×10^7	8.9×10^6	-	-
		39	2.4×10^3	2.3×10^3	2.7×10^7	6.0×10^7	-	-
		50	$<10^1$	$<10^1$	2.0×10^7	2.8×10^7	-	-
10^4		13	4.0×10^2	$<10^1$	2.0×10^6	9.9×10^7	-	-
		26	$<10^1$	$<10^1$	2.8×10^7	1.1×10^7	-	-
		39	$<10^1$	9.0×10^1	4.2×10^7	5.5×10^7	-	-
		50	$<10^1$	$<10^1$	8.4×10^7	4.6×10^7	-	-
10^2		13	1.1×10^1	$<10^1$	3.9×10^6	2.5×10^6	-	-
		26	$<10^1$	$<10^1$	2.5×10^7	7.6×10^7	-	-
		39	$<10^1$	$<10^1$	1.3×10^7	1.5×10^7	-	-
		50	$<10^1$	1.5×10^2	3.3×10^7	3.6×10^7	-	+
10^6	100	28	5.0×10^2	5.0×10^2	6.0×10^6	6.3×10^6	-	-
		56	1.0×10^3	5.0×10^2	8.0×10^6	1.1×10^7	-	-
		84	2.0×10^2	3.0×10^2	5.0×10^7	2.0×10^7	-	-
		112	2.0×10^1	2.0×10^1	3.0×10^7	4.2×10^7	-	-
10^4		28	$<10^1$	$<10^1$	1.5×10^6	3.8×10^6	-	-
		56	$<10^1$	$<10^1$	1.3×10^7	3.4×10^7	-	-
		84	$<10^1$	$<10^1$	8.3×10^8	1.6×10^7	-	-
		112	$<10^1$	$<10^1$	1.4×10^8		-	-
10^2		28	$<10^1$	$<10^1$	2.7×10^6	1.6×10^6	-	-
		56	$<10^1$	$<10^1$	2.2×10^8	3.8×10^7	-	-
		84	$<10^1$	$<10^1$	2.1×10^7	9.0×10^8	-	-
		112	$<10^1$	$<10^1$	8.0×10^7	1.3×10^8	+	-
10^6	200	56	$<10^1$	$<10^1$	1.0×10^5	3.6×10^6	-	-
		112	$<10^1$	$<10^1$	1.0×10^5	1.5×10^5	-	-
10^4		56	$<10^1$	$<10^1$	3.0×10^5	2.5×10^6	-	-
		112	$<10^1$	$<10^1$	1.0×10^5	1.0×10^5	+	-
10^2		56	$<10^1$	$<10^1$	7.0×10^5	2.3×10^6	-	-
		112	$<10^1$	$<10^1$	2.1×10^5	1.5×10^5	-	-

**Report 111. IDENTIFICATION OF SPOILAGE FLORA OF IRRADIATION
PASTEURIZED FISH**

SUMMARY

Due to various factors the group of bacteria causing spoilage in all types of vacuum packed samples were not identified. The identified groups are indicated in the following:

Irradiation Dose (krad)	Storage Temperature (°F)	Spoilage Flora	No. of Isolates Carried Through Biochemical, Micro- scopic and other tests
100	50	not identified	-
	45	50% <u>Lactobacillus</u>	28
		50% <u>Aeromonas</u>	29
	42	50% probably <u>Aeromonas</u>	45
		30% <u>Lactobacillus</u>	36
		10% <u>Bacillus</u>	9
200	38	not identified colony types as at 42°	-
	50	<u>Bacillus</u>	25
	45	not identified	-
	42	<u>Lactobacillus</u>	15
	38	<u>Pediococcus</u>	15

METHODS

Bacterial Isolations from Spoiled Product and Identification of Spoilage Flora

From Eugon and APT agar plate cultures made from spoiled haddock samples, colonies were transferred to APT broth and incubated 24 to 48 hours at 68°F after which they were transferred

to APT or Nutrient (containing 5% of yeast extract and 5% of trypticase) agar slants, allowed to grow at 68°F and then held at 40°F until tests for identification were made.

Tests to identify the family or generic type of the bacteria isolated from spoiled fish were made according to the outline given in table 8. It was not possible to identify the bacteria from all treatments due to the fact that the project ran for only one year and because of the magnitude of the work involved in the identification of large numbers of microorganisms.

No strict anaerobes were isolated among the spoilage flora of haddock vacuum packed and irradiated. It should also be pointed out that while the flora of the 200 krad irradiated samples stored at 38 or 42°F were examined, the organisms identified are not necessarily the spoilage flora since the samples were not spoiled when examined. However, the spoilage flora might not change greatly since the plate count at the time of identification was higher than 10×10^6 per gram.

The following characteristics were recorded for the isolated spoilage flora:

Irradiated at 100 krad

Stored at 50°F - not identified

Stored at 45°F - 50% gram positive, catalase negative rod, facultative to oxygen, ferment sugars - Lactobacillus.

- 50% gram negative rods, catalase positive facultative to oxygen, nitrate positive, oxidase negative, ferments glucose and lactose, alkaline over acid on triple

sugar iron (TSI) agar - probably Aeromonas.

Stored at 42°F - 50% gram negative rods with polar flagella, facultative to oxygen, oxidase negative, nitrate positive, ferments glucose and lactose, alkaline over acid on TSI agar Aeromonas.

- 30% gram positive, catalase negative rods, facultative to oxygen, ferments sugars - Lactobacillus.

- 10% gram positive rods, spore forming, catalase positive, facultative to oxygen - Bacillus.

Stored at 38°F - not identified

Irradiated at 200 krad

Stored at 50°F - 100% gram positive rods, spore forming, catalase positive, facultative to oxygen - Bacillus.

Stored at 45°F - not identified

Stored at 42°F - 96% gram positive, catalase negative rods in pairs, ferment glucose and lactose, facultative to oxygen, acid on TSI agar - Lactobacillus.

Stored at 38°F - 100% gram positive, catalase negative, coccoid-shaped cells, acid reaction on TSI agar - Pediococcus.

TABLE 8.
Gram-Negative Microorganisms

TABLE 8. (continued)

Gram-Positive Microorganisms:

Report IV. MISCELLANEOUS FINDINGS IN RELATION TO THE GROWTH
OF TYPE E CLOSTRIDIUM BOTULINUM IN FISH TISSUES.

SUMMARY

The fat and moisture content of haddock, ocean perch, and mackerel were determined, the results indicating that haddock is low in fat (av. 0.1%), ocean perch somewhat higher in fat (av. 2.5%), and mackerel very high in fat (av. 20%). The moisture content of mackerel was comparatively low (60% as compared to 78-79% for the others).

In fish tissues treated with one megarad and inoculated with spores of type E *Clostridium botulinum* then held at 50°F under aerobic conditions, significant growth occurred in mackerel and ocean perch in 7 days, and there was a considerable increase in numbers in 10 days. The number of cells did not increase significantly in haddock in 10 days. In fish tissues treated as indicated but incubated anaerobically the cells increased to a large number in 10 days and toxin was present in 5 to 7 days in both ocean perch and mackerel. In haddock tissues the cells decreased to much smaller numbers than that of the inoculum in 13 days, and no toxin was produced in 14 days.

Vitamins, A, D, E, various B vitamins and fat extracted from ocean perch were added to irradiation sterilized haddock tissues after which this material was inoculated with 10^3 spores per gram of type E *Clostridium botulinum* and incubated at 50°F under anaerobic conditions. Growth and toxin production were determined during storage on the basis of one series of tests. Both outgrowth of type E *Clostridium botulinum* and

toxin production in haddock were stimulated slightly by the addition of fat extracted from ocean perch and by the addition of vitamin B₁₂.

The effect of added glucose and/or ribose on the growth and toxin by type E *Clostridium botulinum* in haddock tissues has not been determined. It also has not been found that free glucose was higher in ocean perch than in haddock. However, free ribose was higher in ocean perch than in haddock. Ocean perch supported the outgrowth and toxin production of *Clostridium botulinum* type E in a shorter time than did haddock.

Nonheat-shocked spores of type E *Clostridium botulinum*, containing some vegetative cells, were washed, suspended in physiological saline, and held at 50°F. Counts to determine growth and toxin determinations were made periodically. Suspensions of approximately 10^3 cells per ml appeared to show no growth during a period of 42 days. Suspensions containing 10^6 cells per ml were toxic in the trypsinized state after 2 days, and there was an increase in the toxin concentration during storage. After 2 days the highest concentration of toxin indicated the 1:60 dilution to be toxic, and this was the case only in the trypsinized state. After 42 days of storage both the trypsinized and the nontrypsinized suspension were toxic in the 1:1000 dilution.

Nonheat-shocked spores and cells in buffer suspension at a level of approximately 10^5 per ml were shown to be toxic to mice (injection of 0.5 ml) at the start and after trypsinization for various periods up to 150 minutes. There was a

slight decrease in toxicity after 70 minutes of trypsinization. The heat-shocked suspension, handled in the same manner, was not toxic at the start or at any time during trypsinization for periods up to 150 minutes.

The results indicate that in tests to determine toxin formation with fish inoculated with type E *Clostridium botulinum* the use of nonheat-shocked spores as inocula may cause the fish to be toxic because of the presence of toxin already present in the spores and cells, hence may cause the finding of spurious results.

METHODS

Fat and Moisture in Fish Tissues

The fish tissues were ground and mixed after which 10 to 20 gram samples were weighed into aluminum dishes and placed in an oven at 140°F under a vacuum of 25 to 26 inches of mercury. Samples were dried in this manner for 12 to 18 hours then cooled and reweighed on an analytical balance. Moisture contents were calculated according to differences. Freeze-dried samples were hydrated (80% water added) and blended for 2 minutes with a 2:1 mixture of methanol-chloroform. The mixture was then filtered and the residue washed with chloroform. The chloroform layer was removed by means of a separatory funnel, placed in a weighed flask, the chloroform evaporated, and the flask reweighed (1).

Growth of Type E *Cl. botulinum* and Toxin Production in Fish Tissue

Skinless fillets of haddock, ocean perch, and mackerel were ground and mixed after which 20 to 30 gram portions were placed in tubes or plastic bags and then treated with a gamma ray dose of 1×10^6 rad. All samples were then inoculated with spores of the Beluga strain of type E *Cl. botulinum* at a level which provided a concentration of 3.7×10^3 spores per gram in the samples. All samples were now incubated at 50°F, the tubes being incubated anaerobically under hydrogen and carbon dioxide.

Counts for clostridia at 2 day intervals were made on TPG agar with 5% yeast extract containing 0.05% sodium sulfite and 0.07% iron citrate, in Miller-Prickett tubes. The agar

was stratified with vaspar. Cultures were incubated at 30°C for 18 to 48 hours prior to making counts for clostridia as black colonies.

Tests for toxicity were made periodically by extracting the samples (1 part fish to 2 parts of gel-phosphate buffer, pH 6.2) holding the mixture overnight at 33°F, centrifuging and injecting protected and unprotected mice intraperitoneally with 0.5 ml of the trypsinized and nontrypsinized extract, then observing the mice for symptoms of botulism and death.

Effect of Additives on Growth and Toxin Production by type E C. botulinum in Fish Tissues

Haddock tissues were ground and mixed, and 20 gram samples were placed in test tubes then sterilized with a gamma ray dose of 4 megarads. Various vitamins or fat extracted from ocean perch were added to the samples in such concentrations as to approximate the content of the materials as found naturally in herring (vitamins) or the fat content of ocean perch. The amounts of the additives used per 100 grams were as follows:

Vitamin A	150 IU
Vitamin D	300-1700 IU
Vitamin E	146 ug
Vitamin B ₁	0.06 mg
Vitamin B ₂	0.27 mg
Vitamin B ₆	4.5 mg
Vitamin B ₁₂	10 ug
Niacin	40 mg
Pantothenic acid	10 ug
Fat	2.5 g

The samples were inoculated at a level of approximately 1×10^3 spores per gram with the Beluga strain of type E *Cl. botulinum* then incubated at 50°F under anaerobic conditions as previously described. Bacterial counts for clostridia and

tests for toxin were made periodically in the usual manner.

Determination of Free Sugars in Fish Tissues.

Portions of 50 grams of the ground irradiated (4 megarads) or nonirradiated fish tissues were blended for 2 minutes with 100 ml of 10% Trichloroacetic acid. The material was centrifuged and the supernatant washed four times with 50 ml of anhydrous ethyl ether. The samples were then deionized by passing through cation and anion exchange columns (Dovex-3, 20-50 mesh and Amberlite-IR-120, 20-50 mesh). The samples were then concentrated by freeze drying.

Sugars were then separated by paper chromatography using Whatman No. 1 paper and a 4:1:5 mixture of n-butanol, acetic acid, and water as a solvent (2). The spraying agent consisted of an aniline-phthalic mixture (0.93 g aniline + 1.9 g phthalic acid dissolved in 100 ml of butanol (3)).

For the quantitative determination of sugars, extraction from the chromatograph paper was carried out by placing the section of paper containing the particular sugar in distilled water and heating for 15 minutes in a water bath. Glucose was then determined quantitatively by the glucose oxidase method (4). Ribose was determined quantitatively by the orcinol method (5).

DISCUSSION

Fat and Moisture Content of Fish

The fat and moisture content of haddock, ocean perch, and mackerel are shown in Table 9. This investigation was made

to establish the fact that ocean perch, in which type E *Cl. botulinum* appears to grow better than in haddock, is a medium fat fish as compared to haddock, or a low fat fish as compared to mackerel. Correspondingly, the moisture content was highest in haddock and lowest in mackerel.

Growth of type E *Cl. botulinum* in Low, Medium and High Fat Fish

The growth of type E *Cl. botulinum* in preirradiated (one megarad) haddock, ocean perch, and mackerel under anaerobic and under aerobic conditions is shown in figures 2 and 1; and toxin production is shown in Table 10. It is evident that concerning the growth and toxin production by type E *Cl. botulinum*, mackerel is a much better medium than ocean perch and that ocean perch is a much better medium than haddock. Growth and toxin production was best under anaerobic conditions although no toxin was present in haddock samples handled in this manner after 19 days of incubation at 50°F.

Aerobic growth and toxin production was complicated by the fact that organisms other than type E *Cl. botulinum* grew and decomposed the samples.

Effect of Additives on Outgrowth and Toxin Production of Type E *Cl. botulinum* in Haddock Tissues

The growth of type E *Cl. botulinum* in haddock tissues to which vitamins or fat from ocean perch had been added or in haddock or halibut with no additives, incubated anaerobically at 50°F, is shown in figure 3; and the results of toxicity tests on this material are shown in Table 11.

It can be seen from figure 3 that in 28 days in plain haddock or that containing added A, D and E, B₂ or B₆ vitamins

the clostridial count had increased only from 1×10^3 to less than 1×10^4 . Haddock containing added fat from ocean perch increased in clostridial count from 1×10^3 to 1×10^5 and that containing added B_{12} increased in clostridial count from 1×10^3 to 4×10^5 during the same period. Halibut proved to be a better substrate than any of the other samples in supporting the growth of type E *Cl. botulinum*.

Toxicity tests confirmed the findings of the clostridial counts, toxin occurring in haddock samples to which ocean perch fat or vitamin B_{12} had been added and in halibut 4 days earlier than it was present in other samples.

Sugars in Irradiated and Nonirradiated Haddock and Ocean Perch.

Since it has been found (6) that the presence of glucose or ribose may be the reason why certain fish support the growth of type E *Cl. botulinum* to a greater extent than others, tests for these compounds were made in ocean perch and haddock prior to and subsequent to irradiation with 4 megarad of gamma rays. It should be pointed out that ocean perch is a better substrate for the growth of type E *Cl. botulinum* than is haddock. The results of such tests are shown in Table 12.

If both glucose and ribose stimulate growth and toxin production of type E *Cl. botulinum* in fish, this would seem not to be the reason why ocean perch provides a better substrate than haddock, since while ribose is higher in ocean perch than in haddock, glucose is higher in haddock than in ocean perch; and there is little difference in the combined amounts of these sugars in the two fish.

Irradiation at high dose levels (4 megarad) increases the amounts of both sugars in both of these fish. Increases in sugars in these fish irradiated at 100 or 200 krad are now being determined.

Growth of Nonheat Shocked type E *Cl. botulinum* Cells and Spores When Held in Ringer's Solution and Toxicity of Suspensions of Cells and Spores of this Organism.

Growth of type E *Cl. botulinum* in Ringer's solution during storage at 50°F is shown in figure 4, and toxin tests are shown in Table 13. The results of toxin tests on heat shocked and nonheat shocked spores and cells of type E *Cl. botulinum* in gel-phosphate buffer are shown in Table 14. In Ringer's solution concentrations of 1×10^4 and 1×10^6 spores and cells per ml were used while spores and cells in gel-phosphate buffer were present in a concentration of approximately 1×10^5 per ml.

It can be seen from Table 14 that in concentrations of 1×10^5 spores and cells per ml, nonheat shocked cells are toxic in the trypsinized state while heat shocked cells are not toxic.

It is indicated in figure 4 that when nonheat shocked and cells are held in Ringer's solution for long periods at 50°F there may be some growth when they are originally present in high concentration. This is further confirmed by a slight increase in the toxicity of these suspensions during holding (Table 13). It should be noted that these suspensions contained about 30 per cent of vegetative cells.

These findings indicate that in packs of fish inoculated with type E *Cl. botulinum*, irradiated or not irradiated, where the purpose is to determine outgrowth and toxin production, only heat-shocked spores should be used. If this is not done, results positive for toxin may indicate only the toxin already present in the inoculum rather than outgrowth and toxin production by the organism.

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TABLE 9. Fat and Moisture Content of Haddock, Ocean Perch,
and Mackerel.

Fish	Sample No.	Fat Content (Per Cent)	Moisture Content (Per Cent)
Haddock	1	0.38	79.5
	2	0.32	79.4
	3	0.95	79.6
	4	0.02	79.2
	5	0.02	79.2
	6	0.02	79.4
	Averages	0.10	79.4
Ocean Perch	1	1.47	79.5
	2	1.83	79.5
	3	1.49	79.8
	4	3.68	77.6
	5	4.85	77.0
	6	2.86	73.5
	Averages	2.90	78.5
Mackerel	1	26.6	58.9
	2	21.2	55.6
	3	20.4	60.4
	4	17.9	64.6
	5	18.6	63.8
	6	21.4	61.2
	Averages	20.0	60.0

TABLE 10. Toxin in Haddock, Ocean Perch, and Mackerel Samples
Inoculated with type E Cl. botulinum, Beluga Strain,
and Incubated at 50°F.

Fish	Type of Incubation	Toxicity after (days of incubation)						
		<u>2</u>	<u>5</u>	<u>7</u>	<u>10</u>	<u>13</u>	<u>16</u>	<u>19</u>
Haddock	aerobic*	-	-	-	-	-	-	-
	anaerobic	-	-	-	-	-	-	-
Ocean Perch	aerobic*	-	-	-	-	-	-	-
	anaerobic	-	-	-	+	+	+	+
Mackerel	aerobic*	-	-	-	-	-	-	-
	anaerobic	-	-	+	+	+	+	+

* No toxin present, but the fish was badly decomposed,
apparently from the growth of other organisms than type
E Cl. botulinum.

TABLE 11. Toxicity Development in Haddock or Halibut Containing Additives, Inoculated with Type E C1. botulinum and Incubated Anaerobically at 50°F.

Material Added	Fish Tissues Used	Toxicity when Tested after:					
		2 days	5 days	15 days	20 days	24 days	28 days
none	Haddock	-	-	-	-	+	+
Vitamins A,D, & C	Haddock	-	-	-	-	+	+
Vitamin B ₂	Haddock	-	-	-	-	+	+
Vitamin B ₆	Haddock	-	-	-	-	+	+
Vitamin B ₁₂	Haddock	-	-	-	+	+	+
Fat from Ocean Perch	Haddock	-	-	-	+	+	+
none	Halibut	-	-	-	+	+	+

TABLE 12. Glucose and Ribose Content in Haddock and Ocean Perch before and after Irradiation with Gamma Rays at a Level of 4 megarad.

Fish	Sample No.	Non-irradiated		After Irradiation (4.mrad)	
		Sugar	Amount/100g	Sugar	Amount/100g
Haddock	1	Glucose	9.5 mg		
	2	Glucose	18.0 mg		
	3	Glucose	12.5 mg		
		Ribose	3.4 mg		
	4	Glucose	28.0	Glucose	37.5

Ocean Perch	1	Ribose	5.0		
	2	Ribose	25.0 mg		
	3	Ribose	13.5		
	4	Glucose	1.72	Glucose	24.4
	5	Ribose	22.0	Ribose	60.0

TABLE 13. Toxin in Nonheat Shocked Spores and Cells of Type E Cl. botulinum during holding in Ringer's Solution at 50°F.

Incubation days	<u>Dilution Tested</u>				
	6×10^0	3×10^1	6×10^1	3×10^7	1×10^3
2	++	++ --	-- --		
4	++	++ --			
6	++	++ --			
8		++ --			
10		++ --			
12		++ --			-- nontrypsinized ++ trypsinized
14		++ --	++ --		
<hr style="border-top: 1px dashed black;"/>					
42		++ ++	++ ++	++ ++	++ +-

Slight increase of toxicity can be noticed after 12 days of incubation. After 42 days of incubation toxicity has increased 15x as compared with the toxicity level after 14 days of incubation @ 50°F.

TABLE 14. Toxicity of Heat-Shocked Nonheat-Shocked Cells
Type E C1. botulinum Approximately 10^5 Cells/Ml
0.5 ml Injected.

<u>Trypsinized</u> <u>(Min.)</u>	<u>Heat-Shocked</u> <u>No. Positive Samples</u>	<u>Nonheat-Shocked</u> <u>No. Positive Samples</u>
0	0/6	6/6
5	0/6	6/6
10	0/6	6/6
20	0/6	6/6
30	0/6	6/6
40	0/6	6/6
50	0/6	6/6
60	0/6	6/6
70	0/6	5/6
80	0/6	6/6
90	0/6	5/6
100	0/6	4/6
110	0/6	5/6
120	0/6	6/6
130	0/6	3/6
140	0/6	2/6
150	0/6	4/6

Incubation of Haddock, Ocean Perch and Mackerel
inoculated with 10^4 spores of C. botulinum type E in anaerobic conditions at 50° F.

Graph #1

Incubation of Haddock, Ocean Perch and Mackerel inoculated with 10^3 spores of C. botulinum type E in anaerobic conditions at 50°F.

Graph #2

Influence of vitamins addition on growth and toxin production of *Cl. botulinum* type E in Haddock; growth of *Cl. botulinum* type E in Halbut. Anaerobic incubation at 50°F.

Graph #3

Incubation of *Cl. botulinum* type E
in Ringer's Solution and in Haddock; heat shocked
and non-shocked cells; incubation temp. 50°F.

Graph #4