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THE EFFECT OF GAMMA RAYS ON
OCEAN PERCH INOCULATED WITH
TYPE E, CLOSTRIDIUM BOTULINUM

Annual Report, June 1966 — June 1967

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THE EFFECT OF GAMMA RAYS ON OCEAN PERCH INOCULATED WITH
TYPE E, CLOSTRIDIUM BOTULINUM

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Report I   TOXIN PRODUCTION BY TYPE E, CLOSTRIDIUM
BOTULINUM IN THE PRESENCE OF PSEUDOMONAS
FRAGI OR ACHROMOBACTER SPP. IN TPG-HADDOCK
BROTH

SUMMARY

Past experiments have shown that radiation-sterilized haddock inoculated with cells of Ps. fragi (a typical fish spoilage organism) together with heat-shocked spores of type E, Cl. botulinum became toxic after ten days of aerobic incubation at 45°F, while similar samples inoculated with Achromobacter species (radiation-pasteurized fish spoilage organisms) together with the spores of type E, Cl. botulinum became toxic in twenty to thirty days at 45°F (1). In order to confirm the toxicity time for samples inoculated with Ps. fragi and type E, Cl. botulinum, a third series of experiments was run. As with past results these samples became toxic in ten days at 45°F.

Tests were made for the toxicity of TPG-haddock broth inoculated with Ps. fragi and type E, Cl. botulinum or with Achromobacter species and type E, Cl. botulinum incubated aerobically at 45°F. The fragi-botulinum-inoculated samples became toxic in fifteen to twenty days and the Achromobacter-botulinum-inoculated samples became toxic in twenty to 25 days. It is indicated, therefore, that fresh fish spoilage types of bacteria may enhance the outgrowth and toxin production of type E, Cl. botulinum to a greater extent than do those bacterial types which eventually cause spoilage of radiation-pasteurized fish.

METHODS AND PROCEDURES

Preparation of Irradiation-Sterilized Haddock Tissues Inoculated with Spores of Type E, Cl. Botulinum and with Ps. Fragi or Achromobacter

Haddock was ground and 50 g portions placed in polyethylene bags and irradiated at a level of 4 megarad.

After holding for 48 hr at 36 to 40°F, the samples were inoculated with spores of type E, Cl. botulinum (Detroit strain) and with cells of either Ps. fragi or Achromobacter in concentrations that contained about 1×10^4 spores of type E, Cl. botulinum, and about 1×10^4 cells of either Ps. fragi or Achromobacter per gram of tissue.

The inoculum was mixed with the fish by kneading, and the bag contents were incubated aerobically at 45°F. Tests for toxin and for aerobic and clostridial counts were made at five day intervals in the usual manner (1).

Preparation of Broth Cultures of Type E, Cl. Botulinum with Ps. Fragi or Achromobacter

Broth made up with a haddock extract (1 pound of ground haddock boiled in 1 liter of water, then filtered through 3 layers of cheese cloth) to which trypticase, peptone, and glucose were added but no thioglycollate, sodium sulfite, or ferric citrate, was inoculated with spores of type E, Cl. botulinum (Detroit strain) and cells of either Ps. fragi or mixed Achromobacter strains (obtained from Dr. J.S. Lee of Oregon State College, who isolated these Achromobacter species from spoiled, irradiated Dover sole). In inoculating the broth, concentrations of spores and cells were used such that approximately 1×10^4 spores and 1×10^4 cells per ml of broth were provided.

The cultures were then incubated aerobically at 45°F and tested for toxin and for aerobic and clostridial counts at five day intervals.

Aerobic counts were made by surface plating on Eugon agar and by incubating for five days at 68°F. Clostridial counts were made on TPG agar containing 0.05%

ferric citrate, 0.2% sodium thioglycollate, and 0.07% sodium bisulfite stratified with 2% agar containing thioglycollate in Miller-Prickett tubes. Cultures were incubated for 48 hr at 86°F. Black colonies were counted as clostridia.

To determine toxicity, trypsinized and non-trypsinized portions were injected intraperitoneally into unprotected mice and into mice protected with type E, Cl. botulinum antiserum. Two protected and three unprotected mice were injected with 0.5 ml of the 1:10 dilution of the trypsinized cultures, while one protected and two unprotected mice were injected with the 1:10 dilution of the non-trypsinized cultures. The mice were observed for symptoms and death over a period of 72 hr in the usual manner.

DISCUSSION OF RESULTS

Growth of Ps. Fragi and Type E, Cl. Botulinum in Haddock Tissues

The results of tests to determine toxicity in previously radiation-sterilized haddock tissues inoculated with cells of Ps. fragi together with spores of type E, Cl. botulinum are shown in Table 1. As with past results in tests of this kind (1) the samples became toxic in ten days at 45°F. This is two to three times as fast as that period at which toxin develops in haddock tissues otherwise handled in the same manner but inoculate with Achromobacter species together with type E, Cl. botulinum. Ps. fragi is a typical fresh fish spoilage organism, whereas the Achromobacter species were obtained from Dr. J.S. Lee of Oregon State as the typical spoilage organism of irradiated Dover sole. It is implied, therefore,

that fresh fish spoilage bacteria may enhance the outgrowth of type E, Cl. botulinum in haddock tissues held at 45°F, so far as the period required for toxin production is concerned.

Growth of Ps. Fragi or Achromobacter Species with Type E, Cl. Botulinum in TPG-Haddock Broth

The results of tests to determine outgrowth of type E, Cl. botulinum in TPG-haddock extract broth, when grown with either Ps. fragi or Achromobacter species, are shown in Table 2. These results confirm those obtained with haddock tissues, in that a shorter incubation time was required for toxin production with type E, Cl. botulinum and Ps. fragi than with type E, Cl. botulinum and Achromobacter species. The reason for this may be that when cultures or fish tissues are incubated aerobically, as were the samples under investigation, Ps. fragi grows at a faster rate and thus establishes anaerobic conditions to provide for the growth of type E, Cl. botulinum in a shorter time than is the case when Achromobacter are the species which must grow to establish anaerobic conditions.

TABLE 1. Effect of Growth of Ps. fragi on Outgrowth and Toxin Development by Type E, Cl. botulinum in Previously Irradiation-Sterilized Haddock Tissues Stored at 45°F.

Storage Time (days)	Aerobic Count (cells per gram)	Anaerobic	Toxicity
0	1.6×10^3	2×10^2	-
1	2.8×10^4	$< 10^1$	-
5	7.9×10^6	10^1	-
10	1×10^8	1×10^4	+
20	1.9×10^8	1.5×10^4	+
30	1.9×10^8	3.1×10^5	+

+ indicates that the sample was toxic in the 1:10 dilution.
 - indicates that the sample was not toxic in the 1:10 dilution.

TABLE 2. Outgrowth and Toxicity in TPG-Haddock Extract Broth Inoculated with Achromobacter or Ps. fragi and Type E, Cl. botulinum Stored at 45°F (7.2°C) under Aerobic Conditions.

Storage Time (Days)	Clostridia	Aerobes	Toxicity
<u>Achromobacter + strain 8E</u>			
log ₁₀ no. bacteria			
0	4.5	4.6	not done
5	4.4	7.4	-
10	3.6	6.6	-
15	4.0	7.5	-
20	3.8	7.5	-
25	3.3	7.5	+
30	4.3	7.9	+
<u>Ps. fragi + strain 8E</u>			
log ₁₀ no. bacteria			
0	4.3	5.0	-
5	3.3	9.1	-
10	4.0	9.2	-
15	4.0	9.9	+
20	4.6	9.5	+
25	3.5	9.8	not done
30	not done	not done	not done

+ = toxic in the 1:10 dilution; - = not toxic in the 1:10 dilution

* = one of three mice receiving trypsinized extract died; none receiving non-trypsinized extract died.

** = two of three mice receiving trypsinized extract died; none receiving non-trypsinized extract died.

Report II   DESTRUCTION OF TYPE E, CLOSTRIDIUM BOTULINUM
TOXIN IN FISH FILLETS BY COOKING

SUMMARY

Tests made with haddock fillets inoculated with 1×10^6 spores per gram of type E, Cl. botulinum, irradiated at 200 krad and held at 75°F for ten days showed that when activated with trypsin, these fillets contained about 5,000 LD₅₀ mouse units of toxin per 0.5 g. Such fillets were cooked to the underdone stage (3 min in deep fat at 375°F or 3 min per side in pan frying at 400°F), and after this treatment did not contain sufficient toxin to kill any of the mice injected intraperitoneally with the trypsinized or non-trypsinized extract. Cooking, therefore, must be considered a considerable safety factor in regard to any type E botulism public health hazard which fresh or radiation-pasteurized fish presents. No one in the United States eats uncooked fish of the type which would be subjected to radiation-pasteurization treatment.

METHODS AND PROCEDURES

Toxin Tests on Haddock Fillets Inoculated with Type E, Cl. Botulinum Irradiated, Incubated, and Cooked

A number of haddock fillets, 3/4" in maximum thickness, were inoculated with approximately 1×10^6 spores per gram of type E, Cl. botulinum (Beluga strain), packaged in a "Skotch-Pak" bag, and irradiated with a 200 krad dose of gamma rays. They were then stored at 75°F for 10 days.

Four of the incubated fillets were then selected at random, extracted in gel-phosphate buffer (1 portion fish to 2 of buffer), and tested for toxin in the trypsinized and non-trypsinized states. The concentration of toxin was found to be approximately 10,000 LD₅₀

mouse units per 0.5 g of product, non-trypsinized, and 100 to 1000 LD₅₀ mouse units per 0.5 g of product, trypsinized.

The remaining fillets were held overnight at 33°F and then cooked according to the following procedures:

- a) deep fat fried for 3, 4, or 5 min in oil at 375°F, or
- b) pan fried at 400°F for 3, 4, 5, or 6 min on a side.

Immediately after cooking all samples were allowed to air-cool on a paper towel covered plate for 3 min, then were plunged into twice the weight of chilled gel-phosphate buffer (pH 6.2) and blended for 2 min. The homogenate was placed in a refrigerator for one to two hours, centrifuged, and then 1/2 ml portions of the trypsinized and non-trypsinized extracts were injected IP into mice, and the mice observed for symptoms over a period of 48 hr. Duplicate samples of deep fat fried product were examined for all cooking times. In the case of pan fried products, single samples were examined except for the 5 min per side frying time, in which case duplicate samples were examined.

RESULTS AND DISCUSSION

Destruction of Type E, Cl. Botulinum Toxin in Fish Fillets by Cooking

The amounts of toxin formed in fillets inoculated with 1×10^6 spores of type E, Cl. botulinum per gram (mixed spores of Minneapolis and 8E), irradiated with 200 krad and held at 45°F or at 75°F are shown in Table 3. The results of cooking by deep fat frying or by pan frying on the toxicity of fillets inoculated and irradiated as indicated above and held at 75°F for ten days are indicated in Table 4.

It can be seen that in haddock fillets, 50 LD₅₀ mouse units of toxin per 0.5 ml were present in the extract (1 fish to 2 buffer) in 42 days at 45°F. No toxin was present in these samples after 35 days at this temperature. At 75°F the fillets were toxic in three days and 5000 LD₅₀ mouse units of toxin per 0.5 ml of extract were present after ten days.

When fillets treated in such a manner as to contain 5000 LD₅₀ mouse units of toxin in 0.5 ml of the extract (one in three) were undercooked, as by deep fat frying at 375°F for 3 min and air cooling for 3 min, or by pan frying at 400°F for 3 min on each side and air cooling for 3 min, none of the extracts (one to two) were sufficiently toxic to kill mice injected intraperitoneally with 0.5 ml of the extract. It should be noted that mice injected with extracts of products pan fried for 3 or 4 min per side showed some symptoms of botulism but did not die. Both trypsinized and non-trypsinized extracts were used in all cases.

According to the above results it would appear that cooking, even to the underdone state, provides a considerable safety factor with regard to the possibility of type E botulism due to the ingestion of marine products.

TABLE 3. Bacterial Count and Toxicity of Haddock Flesh
Inoculated with Type E Botulinum Spores, Irradiated,
and then Stored at 45 or 75°F.

Storage Time	Bacterial Count		Toxin titer (LD ₅₀ /0.5 ml)
	Clostridia/g	Total Aerobes/g	
Weeks at 45°F			
3	10 ²	19 x 10 ⁶	None
4	10 ²	81 x 10 ⁷	None
5	15 x 10 ⁴	58 x 10 ⁷	None
6	2 x 10 ⁶	32 x 10 ⁷	5 x 10 ¹
Days at 75°F			
0	5 x 10 ³	54 x 10 ¹	None
1	10 ³	50 x 10 ²	None
2	10 ⁴	79 x 10 ⁶	None
3	13 x 10 ⁶	30 x 10 ⁷	5 x 10 ¹
4	27 x 10 ⁶	69 x 10 ⁷	1 x 10 ²
5	70 x 10 ⁶	46 x 10 ⁷	5 x 10 ²
6	50 x 10 ⁶	40 x 10 ⁷	5 x 10 ²
7	40 x 10 ⁶	50 x 10 ⁷	5 x 10 ³
10	20 x 10 ⁶	58 x 10 ⁷	5 x 10 ³

TABLE 4. Toxicity of Haddock Fillets Inoculated with Type E, Cl. botulinum (Beluga Strain), Irradiated at 200 Krad, then Held Ten Days at 75°F and Cooked.

Type of Cooking (frying)	Time of Cooking (min)	Time of Cooling		Toxicity	
		Prior to Extraction (min)		Sample 1	Sample 2
Deep fat	3	3		None	None
Deep fat	4	3		None	None
Deep fat	5	3		None	None
Pan	3/side	3		None*	Not done
Pan	4/side	3		None*	Not done
Pan	5/side	3		None	None
Pan	6/side	3		None	Not done

*The animals had some symptoms of botulism, but did not die.

Report III   LETHALITY OF TYPE E, CLOSTRIDIUM BOTULINUM
TOXIN BY ORAL FEEDING AS COMPARED TO
INTRAPERITONEAL INJECTION

SUMMARY

Tests to compare the lethality of type E, Cl. botulinum toxin in mice fed orally and injected intraperitoneally, have shown that injection is fifty to 100 times more lethal than oral feeding. Since in all tests to determine the toxicity of inoculated (type E, Cl. botulinum) radiation-pasteurization fish fillets, fish fillets, fish tissues, or bacterial cultures were made by intraperitoneal injection of mice, it is evident that the most rigid means of evaluation have been used. It is also probable that many of the samples reported as toxic in the past may not have been considered as such had the tests for toxicity been made by oral feeding of the material being analyzed.

METHODS AND PROCEDURES

Comparison of Oral Toxic Dose and Intraperitoneal Toxic Dose

A TPG-haddock broth in which type E, Cl. botulinum had grown to produce toxin was used as the test medium. Decimal dilutions of the test medium were made with gel-phosphate buffer, and 0.5 ml portions of the trypsinized and non-trypsinized solutions were injected intraperitoneally into groups of six mice or were force-fed to groups of six mice using special intubation needles modified for feeding infant rats (2). The LD₅₀ dose was calculated in the usual manner (3).

RESULTS AND DISCUSSION

Lethality of Type E, Cl. Botulinum Toxin by Oral Feeding as Compared to Intraperitoneal Injection

The number of deaths in groups of six mice fed 0.5 ml of different dilutions of type E, Cl. botulinum toxin

as compared to the number of deaths in groups of six mice injected intraperitoneally with 0.5 ml of the same dilution of toxin are shown in Table 5. When trypsinized toxin was used, the oral LD₅₀ dose was somewhere between the 1:100 and 1:1000 dilution while the intraperitoneal LD₅₀ dose was somewhere between the 1:10,000 and 1:100,000 dilution. Trypsinized toxin was, therefore, approximately 100 times more lethal when injected intraperitoneally than when fed orally. When non-trypsinized toxin filtrates were used, the LD₅₀ intraperitoneal dose was between the 1:10 and the 1:100 dilution while the oral LD₅₀ dose could be considered obtained with the undiluted filtrate. Type E, C1. botulinum, therefore, is at least fifty to 100 times more lethal when injected intraperitoneally than when fed orally. Since all tests to determine toxin in inoculated, irradiated fish and in fish or broth cultures have been tested by intraperitoneal injection of mice and since fish must be consumed orally, it is self-evident that the most exacting methods have been used in testing for toxin.

TABLE 5. Comparison of the Oral Toxicity and Intraperitoneal Toxicity of Type E Botulinum Toxin in Mice.

Route	Dilution factor	No. of deaths/group of six mice	
		Trypsinized	Non-trypsinized
Oral	10^0	6/6	2/6
	10^{-1}	6/6	0/6
	10^{-2}	4/6	0/6
	10^{-3}	2/6	0/6
Intraperitoneal	10^0	-	6/6
	10^{-1}	-	6/6
	10^{-2}	6/6	0/6
	10^{-3}	6/6	0/6
	10^{-4}	5/6	-
	10^{-5}	1/6	-

**Report IV   DETERMINATION OF THE SPOILAGE TIME FOR
RADIATION-PASTEURIZED COD HELD AT DIFFERENT
TEMPERATURES**

SUMMARY

Tests were made with a small expert panel to determine the storage life of cod fillets irradiated at 100 or 200 krad, and held at 38, 42, or 45°F. The results indicated somewhat shorter storage life times than those obtained by the Fish and Wildlife Service using total rejection by an untrained panel as criteria.

METHODS AND PROCEDURES

Determination of Normal Spoilage Time for Skinless Cod Fillets

Skinless cod fillets obtained from two Boston plants were aseptically cut into 100 g portions and placed in polyethylene bags. The bags were twisted and secured with a plastic covered wire, and the excess portion of each bag tied in a knot. They were then placed in metal boxes and irradiated at 100 and 200 krad. After irradiation the bags were placed at 38, 42, or 45°F.

For each storage time, duplicate samples were checked for odor acceptability by a panel of three people. Each sample was then blended 1:2 with gel-phosphate buffer (pH 6.2), serially diluted, and plated on Eugon agar with 1% yeast extract (a comparison of pour plate versus streak plate counts was made at this time). It was found that there was as much as a log cycle difference in the count obtained with the two methods at the beginning of the storage study (the streak plate count being higher); but as the storage time increased and aerobic conditions decreased within the packages, there was very little difference between the count obtained by pour plate and that obtained by streak plate methods.

The normal spoilage time was determined by total rejection by the trained panel on the basis of odor.

RESULTS AND DISCUSSION

Determination of the Spoilage Time for Irradiation-Pasteurized Cod Held at Different Temperatures

A three member trained panel was used to determine spoilage of cod fillets irradiated at 100 or 200 krad and stored at 38, 42, or 45°F. Spoilage time was determined by odor and considered to be the time at which all three testers rejected the samples. The results are shown in Tables 6 and 7. The approximate spoilage times (from data taken from Tables 6 and 7) are summarized as follows:

Spoilage Times for Cod (Days)

Temperature of Storage	100 Krad Treated	200-Krad Treated
38°F	21	24
42°F	15	15
45°F	9	11

These times are one to four days shorter at 45°F and three to ten days shorter at 42°F than those obtained by the Fish and Wildlife Service using total rejection by an untrained panel of more participants. It would appear, then, that spoilage times for untreated and irradiated cod, as determined by the Fish and Wildlife Service, are the absolute limit of time-temperature holding at which anyone, except those races known to like decomposed fish (some American Indians and Esquimaux), would be able to utilize fresh or irradiated cod as food.

It can also be seen from Tables 6 and 7 that at the time of rejection the aerobic plate count for the irradiated product was usually in the 10^8 per gram region but varied in the extremes between 4.3×10^8 and 2.5×10^9 per gram.

TABLE 6. Results for Determination of Normal Spoilage Time for Skinless Cod Fillets from Plant A Irradiated at 100 and 200 Krad and Stored at 45, 42, and 38°F.

Storage Temp.	Rad dose (Krad)	Storage Time (Days)	Aerobic Counts (Streak Plate)		Odor Reactions			
			Sample 1	Sample 2	Tester 1		Tester 2	
					Sample 1	Sample 2	Sample 1	Sample 2
45°F	0	0	1.3×10^5	1.6×10^6				
		0	1.1×10^4	6.2×10^3				
		2	7.0×10^5	1.1×10^6	ok	ok	ok	ok
		4	3.3×10^7	5.1×10^7	ok	ok	ok	ok
		6	4.2×10^8	3.1×10^8	ok	borderline	ok	borderline
		9	4.6×10^8	6.9×10^8	no	no	borderline	no
	200	11	2.5×10^9	1.1×10^9	no	no	--	--
		0	1.8×10^3	2.5×10^3				
		2	2.1×10^4	3.5×10^4	ok	ok	ok	ok
		4	7.2×10^5	2.0×10^5	ok	ok	ok	ok
		6	1.7×10^6	1.4×10^8	ok	ok	ok	ok
42°F	100	9	4.6×10^6	1.7×10^8	ok	ok	ok	ok
		11	5.8×10^8	3.0×10^8	borderline	borderline	--	--
		14	6.7×10^8	5×10^8	no	no	no	no
		4	7.2×10^6	3.0×10^5	ok	ok	ok	ok
		7	1.5×10^8	1.4×10^8	ok	ok	borderline	ok
		11	3.3×10^8	6.5×10^8	no	no	--	--
		15	7.2×10^8	4.3×10^8	no	no	borderline	borderline

TABLE 6. (continued)

Storage Temp.	Rad dose (Krad)	Storage	Aerobic Counts		Odor Reactions			
		Time	(Streak Plate)		Tester 1		Tester 2	
		(Days)	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
38°F	200	4	2.3×10^6	2.8×10^5	ok	ok	ok	ok
		7	8.1×10^7	4.7×10^7	ok	ok	borderline	borderline
		11	2.5×10^8	3.8×10^8	borderline	borderline	--	--
		15	6.2×10^8	2.2×10^8	no	no	no	no
	100	7	9.2×10^7	3.1×10^7	ok	ok	ok	ok
		11	5.8×10^8	2.1×10^8	ok	ok	--	--
		15	4.2×10^8	3.4×10^9	ok	ok	borderline	ok
		18	6.2×10^8	5.9×10^8	no	no	ok	ok
		21	4.8×10^8	4.1×10^8	no	no	borderline	borderline
		24	6.1×10^8	7.0×10^8	--	--	ok	ok
		7	4.6×10^7	3.3×10^7	ok	ok	ok	ok
		11	1.7×10^8	1.7×10^8	ok	ok	--	--
		15	5.4×10^8	4.5×10^8	ok	ok	ok	ok
		18	4.8×10^8	4.4×10^8	no	no	ok	ok
		21	3.8×10^8	6.1×10^8	no	borderline	ok	no
		24	4.3×10^8	5.4×10^8	--	--	ok	ok

TABLE 7. Test Results for Determination of Normal Spoilage Time for Skinless Cod Fillets from Plant B Irradiated at 100 and 200 Krad and Stored at 45, 42, and 38°F.

Storage Temp.	Rad dose (Krad)	Storage Time (Days)	Aerobic Counts (Streak Plate)		Odor Reactions			
			Sample 1	Sample 2	Tester 1		Tester 2	
					Sample 1	Sample 2	Sample 1	Sample 2
45°F	0	0	8.4×10^5	1.1×10^6				
		0	4.2×10^3	5.3×10^3				
		2	1.4×10^6	1.2×10^6	ok	ok	ok	ok
		5	2.4×10^7	2.9×10^7	no	ok	ok	ok
		8	2.6×10^8	3.9×10^8	probably no	no	borderline	borderline
		13	6.3×10^8	9.4×10^8	no	no	no	no
	200	0	3.5×10^2	2.4×10^3				
		2	1.7×10^4	4.3×10^4	ok	ok	ok	ok
		5	7.3×10^7	8.0×10^7	ok	ok	ok	ok
		8	3.3×10^8	4.7×10^8	ok	ok	ok	ok
		13	6.5×10^8	7.1×10^8	no	no	probably no	probably no
	100	4	7.0×10^7	6.8×10^7	ok	ok	ok	ok
		7	2.3×10^8	5.6×10^8	ok	ok	ok	ok
		10	3.7×10^8	5.4×10^8	no	no	--	--
		14	7.2×10^8	4.3×10^8	no	no	no	no

TABLE 7. (continued)

Storage Temp.	Rad dose (Krad)	Storage Time (Days)	Aerobic Counts		Odor Reactions			
			(Streak Plates)		Tester 1		Tester 2	
			Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
42°F	200	4	2.4×10^7	3.2×10^7	ok	ok	ok	ok
		7	6.8×10^7	1.1×10^8	ok	ok	ok	ok
		10	5.8×10^8	1.9×10^8	no	probably no	--	--
		14	9.1×10^8	4.1×10^8	no	no	borderline	borderline
38°F	100	7	1.1×10^8	1.6×10^8	ok	ok	ok	ok
		10	2.2×10^8	1.7×10^8	ok	ok	--	--
		14	1.0×10^9	8.1×10^8	borderline	borderline	ok	ok
		17	5.3×10^8	8.7×10^8	borderline	no	ok	borderline
		20	8.5×10^8	6.7×10^8	no	no	borderline	no
		23	1.0×10^9	6.7×10^8	--	--	ok	no
	200	7	1.5×10^7	4.0×10^7	ok	ok	ok	ok
		10	1.6×10^8	1.2×10^8	ok	ok	--	--
		14	5.0×10^8	5.1×10^8	borderline	borderline	ok	ok
		17	2.4×10^8	2.7×10^8	no	borderline	ok	ok
		20	5.4×10^8	4.2×10^8	no	borderline	borderline	ok
		23	6.0×10^8	7.2×10^8	--	--	no	borderline

Report V

TOXICITY TESTS ON OCEAN PERCH INOCULATED
WITH TYPE E, CLOSTRIDIUM BOTULINUM IRRADIATED
OR NON-IRRADIATED AND HELD AT DIFFERENT
TEMPERATURES

SUMMARY

Toxicity tests were completed for ocean perch inoculated with 1×10^2 or 1×10^4 spores per gram of type E, Cl. botulinum, non-irradiated or irradiated at 150, 250, or 350 krad, and held for various periods at 50, 45, or 40°F. Irradiated samples either contained toxin at the same time as the control or were non-toxic for longer periods than the control except in several instances. In these cases (those inoculated with 1×10^4 spores per gram, irradiated, and held at 50°F) the control (inoculated, non-irradiated) was toxic in six to ten days; the 150 krad irradiated was toxic in ten days; and the 250 and 350 krad irradiated were toxic in seven to nine days. These are equivalent to two times storage life for the control but at least three days under the storage life for the irradiated evaluated on the basis of the Fish and Wildlife Service storage life times for cod. All other toxin development times for the inoculated, irradiated product were beyond the storage life times as determined by the Fish and Wildlife Service for irradiated cod. It is recognized that since no storage life time (irradiated and non-irradiated product) has been determined for ocean perch, the foregoing comparison has no absolute significance.

METHODS AND PROCEDURES

Procedures for Obtaining Spores of Type E, Cl. Botulinum for the Inoculation of Ocean Perch Packs

Beluga, Detroit, Minneapolis, and 8E strains of Type E, Cl. botulinum were inoculated into deaerated 10 ml portions of trypticase (5%)-peptone (0.5%)-glucose (1%) broth with 0.2% sodium thioglycollate, and heat-shocked at 140°F for 13 min. The broth was stratified with vaspar and incubated at 86°F for 18 to 24 hr. The mixture was

then transferred to 90 ml of deaerated broth which was stratified with vaspar and incubated at 86°F for 8 to 10 hr. This was then transferred to 400 ml of deaerated broth, stratified with vaspar, and incubated at 86°F for 18 to 24 hr (until about 80% spores were present). The cultures were transferred to 45°F for about 18 to 20 hr prior to centrifuging for purposes of harvesting spores. The cultures were centrifuged at 8000 rpm for 20 min after which the spore concentrate was washed and centrifuged at 8000 rpm for 15 min. After this was done three times, the spore concentrate was suspended in 20 ml of sterile distilled water and held at 33°F until inoculation.

Inoculation of Ocean Perch

Ten pound packages of ocean perch fillets were inoculated with a mixture of heat-shocked spores (140°F for 13 min) of type E, Cl. botulinum (Minneapolis, Detroit, Beluga, and 8E) at levels sufficient to provide a final concentration of 1×10^2 or 1×10^4 spores per gram in each of the fillets. A 10 ml syringe with a 22 gauge needle was used for inoculating the fillets. The inoculated fillets were placed inside double plastic bags in a wax-coated cardboard carton. The inner bag was heat sealed and the outer bag sealed with a plastic covered wire closure. The packages were irradiated at 0, 150, 250, and 350 krad and stored at 50, 45, and 40°F.

For Run B the method of inoculation was the same except that only mixed spores of two strains of type E, Cl. botulinum (Beluga and 8E) were used for inoculating the ocean perch fillets instead of the four strains used in Run A.

Procedures for Bacterial Counts and Toxin Assay for Inoculated Ocean Perch Fillets

For the clostridial counts trypticase-peptone-glucose agar with 0.05% sodium thioglycollate, 0.05% ferric citrate, 0.07% sodium sulfite, and 0.01% sodium bicarbonate in Miller-Prickett tubes was used. These cultures were stratified with 2% agar containing 0.2% sodium thioglycollate and incubated at 85°F. The pour plate technique with Eugon agar plus 0.5% yeast extract was used for aerobic counts. The plates were incubated for five to six days at 68°F prior to counting.

For the toxin assay, part of the supernatant from the 1:10 blend of the fish sample was trypsinized (1%) for 45 min at 98.6°F (4). Portions of 0.5 ml of the trypsinized and non-trypsinized extracts were injected intraperitoneally into mice, two unprotected and one protected with type E antitoxin. The mice were observed for 48 to 72 hr.

RESULTS AND DISCUSSION

Toxicity Test on Ocean Perch Inoculated with Type E, Cl. Botulinum, Irradiated or Non-Irradiated, and Held at Different Temperatures

The results of toxicity tests on ocean perch inoculated with type E, Cl. botulinum are shown in Tables 8 to 17 inclusive.

It should be noted that there were two different series of tests, Run A and Run B. In all tests fillets were inoculated with heat-shocked spores of type E, Cl. botulinum, and toxicity tests were made by intraperitoneal injection of mice with the trypsinized and non-trypsinized

gel-phosphate buffer extract of the fillets. In Run A, heat-shocked spores of mixed strains (Detroit, Minneapolis, Beluga, and 8E) of type E, Cl. botulinum were used for inoculation while in Run B heat-shocked spores of strains Beluga and 8E were used.

A summary of the results taken from the data in Tables 8 to 17 inclusive is shown below:

Ocean Perch

Storage Temp. (°F)	Run A				Run B			
	Toxicity Time (Days) at Dose Level (Krad)				Toxicity Time (Days) at Dose Level (Krad)			
	none	150	250	350	none	150	250	350
	<u>1 x 10² Inoculum</u>							
50	10	10	14	9	6	10	9	9
45	18	31	26	17				
40	>25	36	30	>56				
	<u>1 x 10⁴ Inoculum</u>							
50	10	10	7	9	6	7	8	8
45	18	24	25	17				
40	>25	>38	>45	>54				

Since heat-shocked spores of four strains of type E, Cl. botulinum were used for Run A and toxicity tests were made on a 1:10 buffer extract—the same conditions as were used for previous tests with haddock (1)—and the toxicity times for irradiated and non-irradiated products are generally shorter than those obtained with haddock, it is indicated that ocean perch is a somewhat better substrate for the growth of type E, Cl. botulinum than is haddock.

In general the irradiated samples did not become toxic in shorter periods than did the controls. However, there were a number of instances in which the toxicity times of controls and irradiated samples were the same or only slightly different. Since irradiated fillets generally have a longer organoleptic storage life than controls there may be some safety problems involved in the radiation-pasteurization treatment of ocean perch.

TABLE 8. Test Results for Ocean Perch Fillets Inoculated with 10^2 Heat Shocked Spores of Type E, Cl. botulinum (Four Strains) and Non-Irradiated (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples		of Samples			
		1	2	1	2	1	2
50°F	5	2×10^2	2×10^2	5.7×10^8	2.8×10^8	-	-
	10	8×10^5	3×10^5	5.6×10^8	9.0×10^8	+	+
45°F	6	1×10^3	1×10^2	1.2×10^8	1.9×10^8	-	-
	12	7×10^4	1×10^4	8.1×10^8	6.4×10^8	-	-
	18	3.8×10^3	3.1×10^3	8.1×10^8	7.1×10^8	+	+
40°F	9	1×10^2	1×10^2	3.1×10^8	2.5×10^8	-	-
	16	$< 10^1$	1×10^2	4.0×10^8	3.3×10^8	-	-
	25	1×10^2	1×10^2	2.1×10^8	5.7×10^8	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 9. Test Results for Ocean Perch Fillets Inoculated with 10^4 Heat Shocked Spores of Type E, Cl. botulinum (Four Strains) and Non-Irradiated (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples	
		1	2	1	2	1	2
50°F	5	5×10^3	$< 10^3$	5.4×10^7	1.5×10^7	-	-
	10	6×10^5	5×10^5	4.4×10^8	4.0×10^8	+	+
45°F	6	2×10^3	2×10^3	1.3×10^8	3.3×10^8	-	-
	12	3×10^4	1.0×10^5	9.7×10^8	5.0×10^8	-	-
	18	6×10^2	8.0×10^4	1.4×10^9	6.1×10^8	+	+
40°F	8	2.0×10^3	5×10^3	2.0×10^8	9.5×10^7	-	-
	16	2×10^3	3.0×10^4	5.3×10^8	3.4×10^8	-	-
	25	2×10^3	1×10^3	1.5×10^8	3.3×10^8	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 10. Test Results for Ocean Perch Fillets Inoculated with 10^2 Heat Shocked Spores of Type E, Cl. botulinum (Four Strains) and Irradiated at 150 Krad (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples	
		1	2	1	2	1	2
50°F	6	6×10^3	2×10^3	7.5×10^7	4.0×10^8	-	-
	10	2×10^4	3×10^4	4.0×10^8	5.3×10^8	-	+
45°F	10	$< 10^1$	$< 10^1$	1.1×10^8	1.5×10^8	-	-
	20	$> 2.5 \times 10^6$	1.0×10^6	7.1×10^7	3.5×10^8	-	-
	31	2.0×10^7	2.2×10^7	9.5×10^8	7.9×10^7	+	+
40°F	12	1×10^2	$< 10^1$	1.6×10^8	1.7×10^8	-	-
	25	2.5×10^4	$< 10^1$	1.9×10^8	4.2×10^8	-	-
	36	$< 10^1$	$< 10^1$	1.9×10^8	3.7×10^8	-	+

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 11. Test Results for Ocean Perch Fillets Inoculated with 10^4 Heat Shocked Spores of Type E, Cl. botulinum (Four Strains) and Irradiated at 150 Krad (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples		of Samples			
		1	2	1	2	1	2
50°F	5	3.0×10^3	1.6×10^3	1.1×10^7	1.2×10^7	-	-
	10	3.0×10^6	4.0×10^6	2.9×10^8	1.9×10^8	+	+
45°F	10	2.0×10^2	1×10^2	5.0×10^8	2.5×10^8	-	-
	24	$>2.0 \times 10^6$	1.9×10^6	3.1×10^8	1.1×10^8	+	+
40°F	12	3×10^2	1×10^2	2.5×10^8	1.5×10^8	-	-
	25	$< 10^1$	3×10^2	8.6×10^7	9.4×10^7	-	-
	38	1×10^1	2.0×10^3	1.8×10^8	6.2×10^8	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 12. Test Results for Ocean Perch Fillets Inoculated with 10^2 Heat Shocked Spores of Type E, Cl. botulinum (Four Strains) and Irradiated at 250 Krad (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples	
		1	2	1	2	1	2
50°F	5	3.1×10^3	5×10^3	8.8×10^7	6.7×10^7	-	-
	14	$>1.0 \times 10^7$	$>1.0 \times 10^7$	4.5×10^8	4.7×10^8	+	+
45°F	13	1.0×10^5	1.5×10^4	2.3×10^8	3.0×10^8	-	-
	26	1.7×10^7	1.9×10^7	3.0×10^8	3.1×10^8	+	+
40°F	15	$< 10^1$	$< 10^1$	2.1×10^8	1.2×10^8	-	-
	30	3.5×10^5	3.5×10^5	6.0×10^8	5.7×10^8	-	+

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 13. Test Results for Ocean Perch Fillets Inoculated with 10^4 Heat Shocked Spores of Type E, Cl. botulinum (Four Strains) and Irradiated at 250 Krad (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		<u>of Samples</u>		<u>of Samples</u>			
		1	2	1	2	1	2
50°F	7	4×10^4	1.0×10^5	6×10^6	6.0×10^7	-	+
45°F	13	1.0×10^5	1.5×10^4	2.3×10^8	3.0×10^8	-	-
	25	1.7×10^7	1.9×10^7	3.0×10^8	3.1×10^8	+	+
40°F	15	1×10^1	8×10^1	1.6×10^8	1.1×10^8	-	-
	30	6×10^1	2×10^1	5.6×10^8	3.6×10^8	-	-
	45	$< 10^1$	4.5×10^1	6.2×10^8	2.7×10^8	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 14. Test Results for Ocean Perch Fillets Inoculated with 10^2 Heat Shocked Spores of Type E, Cl. botulinum (Four Strains) and Irradiated at 350 Krad (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples		of Samples			
		1	2	1	2	1	2
50°F	9	$>1.0 \times 10^4$	$>1.0 \times 10^4$	9.8×10^7	2.3×10^7	+	+
45°F	17	2.0×10^6	3.4×10^6	6.4×10^8	2.0×10^8	+	+
40°F	20	1×10^1	$< 10^1$	1.0×10^8	1.2×10^8	-	-
	36	1×10^1	2×10^1	4.1×10^8	7.0×10^8	-	-
	56	$< 10^1$	$< 10^1$	8.5×10^8	1.6×10^8	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 15. Test Results for Ocean Perch Fillets Inoculated with 10^4 Heat Shocked Spores of Type E, Cl. botulinum (Four Strains) and Irradiated at 350 Krad (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		<u>of Samples</u>		<u>of Samples</u>			
		1	2	1	2	1	2
50°F	9	3.0×10^6	4.0×10^6	1.4×10^8	5.1×10^7	+	+
45°F	17	3.0×10^6	2.0×10^6	1.8×10^8	1.6×10^8	+	+
40°F	17	2×10^2	2×10^3	1.2×10^7	1.4×10^7	-	-
	38	7×10^1	1.6×10^2	6.0×10^8	3.7×10^8	-	-
	54	5×10^2	5×10^2	1.4×10^8	1.5×10^8	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 16. Test Results for Ocean Perch Fillets Inoculated with 10^2 Heat Shocked Spores of Type E, Cl. botulinum (Two Strains), Non-Irradiated or Irradiated, and Stored at 50°F (Run B).

Rad dose (Krad)	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples		of Samples			
		1	2	1	2	1	2
0	6	between 1×10^2 1×10^3	between 1×10^2 1×10^3	4.4×10^8	4.9×10^8	+	-
	7	between 1×10^3 1×10^4	between 1×10^3 1×10^4	5.2×10^8	6.1×10^8	+	-
	8	1.2×10^4	between 1×10^3 1×10^4	7.3×10^9	7.3×10^9	+	+
	9	$>1 \times 10^4$	$>1 \times 10^4$	1.2×10^9	4.5×10^8	not done	not done
150	6	$< 10^1$	$< 10^1$	2.5×10^8	1.5×10^8	-	-
	7	1.2×10^2	4×10^2	5.2×10^8	9.1×10^8	-	-
	8	5.0×10^2	2.0×10^2	6.8×10^8	6.0×10^8	-	-
	9	3×10^2	1.6×10^4	8.0×10^8	6.1×10^8	-	-
	10	4.3×10^6	5×10^6	3.7×10^8	4.6×10^8	+	+
	11	7.3×10^6	3.0×10^6	3.3×10^8	5.4×10^8	+	+

TABLE 16 (continued)

Rad dose (Krad)	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples	of Samples	of Samples	of Samples	1	2
		1	2	1	2		
250	6	1.0×10^2	2.8×10^2	6.3×10^7	9.2×10^7	-	-
	7	3.0×10^4	1.0×10^3	1.2×10^8	1.8×10^8	-	-
	8	2.6×10^4	1.5×10^5	2.9×10^8	2.6×10^8	-	-
	9	1.8×10^4	1.0×10^4	2.9×10^8	4.3×10^8	+	-
	10	1.2×10^6	4.3×10^5	2.2×10^8	2.1×10^8	-	-
	11	2.1×10^7	1.3×10^7	3.5×10^8	3.2×10^8	+	+
350	6	5.0×10^2	1.9×10^2	1.4×10^7	1.9×10^7	-	-
	7	3.4×10^5	2.4×10^4	3.1×10^8	1.2×10^8	-	-
	8	8×10^5	2.2×10^6	3.5×10^7	1.2×10^7	-	-
	9	3.4×10^6	1.3×10^7	5.8×10^7	7.7×10^7	+	+

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 17. Test Results for Ocean Perch Fillets Inoculated with 10^4 Heat Shocked Spores of Type E, Cl. botulinum (Two Strains), Non-Irradiated or Irradiated, and Stored at 50°F (Run B).

Rad dose (Krad)	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples		of Samples			
		1	2	1	2	1	2
0	6	between 1×10^2 & 1×10^3	between 1×10^2 & 1×10^3	1.3×10^8	5.4×10^7	+	+
	7	1.4×10^3	1.8×10^4	4.3×10^8	8.1×10^8	+	-
	8	4.4×10^4	2.7×10^4	2.8×10^9	5.5×10^8	+	+
150	6	3.5×10^4	3.0×10^3	1.4×10^8	1.4×10^8	-	-
	7	$>5.0 \times 10^5$	$>5.0 \times 10^5$	2.5×10^8	3.6×10^8	+	-
	8	5.8×10^6	1.1×10^7	2.7×10^8	4.2×10^8	+	+
250	6	1.2×10^4	1.0×10^4	5.5×10^7	4.8×10^7	-	-
	7	9.1×10^4	1.1×10^5	1.3×10^8	1.3×10^8	-	-
	8	1.4×10^5	1.5×10^5	1.7×10^8	1.2×10^8	+	+

TABLE 17. (continued)

Rad dose (Krad)	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		<u>of Samples</u>		<u>of Samples</u>		<u>of Samples</u>	
		1	2	1	2	1	2
350	6	3.5×10^4	2.0×10^4	3.9×10^7	1.7×10^7	-	-
	7	2.1×10^5	2.6×10^5	8.8×10^7	1.4×10^8	-	-
	8	1.3×10^6	1.6×10^6	6.1×10^8	8.8×10^7	+	-
	9	6×10^6	2.3×10^7	1.3×10^8	8.7×10^7	+	+

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

Report VI   TOXICITY TESTS ON COD INOCULATED WITH
TYPE E, CLOSTRIDIUM BOTULINUM, IRRADIATED
OR NON-IRRADIATED, AND HELD AT DIFFERENT
TEMPERATURES

SUMMARY

Although the tests have not been entirely finished the results of toxicity tests on irradiated and non-irradiated samples of cod inoculated with type E, Cl. botulinum have been completed for all significant storage periods. In one out of three tests (made on duplicate samples), the product inoculated with 1×10^4 spores per gram of type E, Cl. botulinum, irradiated at 100 krad and held at 50°F and irradiated at 200 krad and held at 50°F or 45°F became toxic prior to the time found by the Fish and Wildlife Service as the storage life time for cod irradiated at these levels and held at the temperatures stated.

In inoculating cod with 1×10^2 and 1×10^4 cells per gram, non-heat-shocked cells (for the most part, spores with some cells) have been used. In inoculating cod with 1×10^6 spores per gram of type E, Cl. botulinum, heat-shocked spores have been used. Since cod inoculated with 1×10^6 spores per gram, irradiated at 100 and 200 krad and held at 50°F and 45°F became toxic only after holding for storage periods which were longer than those in which toxicity was obtained in cod inoculated with 1×10^4 cells per gram and otherwise treated in the same manner, the question arises as to whether the use of non-heat-shocked cells has in some way biased the results of toxicity tests. It should also be noted that with cod inoculated with 1×10^6 spores per gram of type E, Cl. botulinum, irradiated at 100 or 200 krad and held at 50, 45, 42, or 38°F, none of the samples became toxic within the storage life times determined by the Fish and Wildlife Service. These storage life times were said to be determined by odor tests involving total rejection by an untrained panel.

Only with the 1×10^4 cells per gram inoculum of type E, Cl. botulinum have irradiated cod samples become toxic after periods within or near the storage life time determined by the Fish and Wildlife Service. It is not considered that this result indicates that irradiation-pasteurized fish represent a type E botulism public health hazard for the following reasons:

(a) In fillets or fish flesh, contamination with type E, Cl. botulinum can only come from the intestinal contents of fish or from fish plant equipment. (b)

Were a sufficient number of cells of type E, Cl. botulinum present in fish intestinal contents to provide an inoculum of 1×10^4 spores per gram in fish flesh, the fish itself would be dead before caught! (c)

Were a sufficient number of cells present on fish plant equipment to provide an inoculum of 1×10^4 cells per gram in fish flesh, toxin would also be picked up by the fish flesh along with the inoculum of cells; hence, under such conditions, the fresh, non-irradiated fish fillets or steaks might be toxic and represent a type E botulism public health hazard. (d) In past tests made in our laboratories to determine the numbers of type E, Cl. botulinum cells present in fish flesh, the highest concentration found was 0.17 per gram; the use of 1×10^4 cells per gram as an inoculum, therefore, represents an entirely unrealistic situation.

METHODS AND PROCEDURES

Procedure for Obtaining Spores of Type E, Cl. Botulinum for the Inoculation of Skinless Cod Fillets

Colonies of Beluga and 8E strains of type E, Cl. botulinum from trypticase-peptone-glucose agar (TPG) plates incubated at 75°F for three days in a Torbal anaerobic jar were each inoculated into 10 ml of deaerated TPG broth containing 1% yeast extract and 0.05% sodium thioglycollate. The broth was stratified with vaspar,

heat-shocked at 150°F for 15 min, cooled immediately, and incubated at 86°F for 18 to 20 hr. From the overnight cultures 1 ml portions were transferred to 10 ml portions of deaerated TPG broth with yeast extract and sodium thioglycollate, stratified with vaspar, heat-shocked at 150°F for 15 min, cooled immediately, and incubated at 50°F for five to seven days. The 10 ml portions were transferred to 90 ml of the same type of deaerated broth, stratified with vaspar, and incubated at 50°F for five to seven days. This mixture was then transferred to 400 ml of deaerated broth, stratified with vaspar, and incubated at 50°F for five to seven days (until about 80% spores were present). The cultures were centrifuged at 8000 rpm for 20 min after which the spore concentrate was washed and centrifuged at 8000 rpm for 15 min. After three times washing and centrifuging, the spore concentrate was suspended in 20 to 30 ml of sterile distilled water, depending upon the amount of sediment, and held at 33°F until inoculation, a period varying between overnight and two weeks.

Procedure for Preparing Spores for Inoculation of Skinless Cod Fillets

Since the levels of 10^2 spores per gram and 10^4 spores per gram of fish were to be inoculated without heat-shocking, the morning of the day before inoculation a portion of the spore suspension held more than two days at 33°F was washed four to eight times (depending upon how long the spore suspension had been held at 33°F) to remove residual toxin. After washing, the spores were re-suspended in 10 ml of sterile distilled water. Heat-shocked and non-heat-shocked counts were made in Miller-Prickett tubes using TPG agar as previously indicated for the new suspension. The following day the new spore suspension was diluted (according to the spore

count obtained by the Miller-Prickett tube technique) to levels sufficient to provide final concentrations of 1×10^2 spores per gram or 1×10^4 spores per gram in each portion of fish.

In one case the spores used for inoculation of 10^2 non-heat-shocked spores per gram were harvested from the TPG culture broth, washed three times in distilled water, the counts of heat-shocked and non-heat-shocked spores done on one day, and the cod fillets inoculated with the desired concentration of spores the next day without further washing. (This applied to Run B: 10^2 non-heat-shocked spores per gram, irradiated at 100 and 200 krad and stored at 50, 42, and 38°F.)

For the level of 10^6 spores per gram of fish, a portion of the spore suspension held at 33°F was heat-shocked (140°F for 13 min) and counted. The next day this suspension was diluted to a level sufficient to provide a final concentration of 1×10^6 spores per gram in each portion of fish.

Inoculation of Skinless Cod Fillets

For Run A of skinless cod fillets inoculated with 1×10^2 non-heat-shocked spores per gram, a lot of commercial cod fillets obtained from a Boston plant were skinned, cut into 100 g portions and each portion placed in a polyethylene bag. Each portion of fish was inoculated with 1×10^2 spores per gram (10^4 spores per cc of inoculum) of a non-heat-shocked suspension of Beluga strain of type E, Cl. botulinum, using multiple jabs with a 10 ml syringe and 22 gauge needle. After inoculation, the packages were sealed with a heat sealer. The packs were then placed in a metal box with a cover for purposes of irradiation at 100 and 200 krad, after which they were placed in storage at 38, 42, 46 and 50°F.

(Note: For this run two separate samples were used for clostridial and aerobic counts and two separate samples for toxin assay.)

For Run A of skinless cod fillets inoculated with 1×10^2 and 1×10^4 non-heat-shocked spores per gram and non-irradiated; for one run of fillets inoculated with 1×10^4 non-heat-shocked spores per gram and irradiated; and for one run of fillets inoculated with 1×10^6 heat-shocked spores per gram and non-irradiated (only 45°F), one lot of commercial cod fillets in the skinless form, obtained from a Boston plant, was aseptically cut into 150 g portions, and each portion placed in a polyethylene bag. Each pack was inoculated with a level of spores sufficient to provide the desired final concentrations per gram in each portion of fish. The fish portions were injected in the same manner as described above. The air was squeezed out of each bag, then the bag was twisted and secured with a piece of plastic covered wire. The excess part of the bag was tied into a knot. The non-irradiated samples were placed in metal boxes and stored at 38, 42, and 45°F. The samples to be irradiated were placed in metal boxes and irradiated at 100 or 200 krad, then stored at 38, 42, and 45°F

One hundred gram portions of skinless cod fillets instead of 150 g portions were used for the following: Run A inoculated with 1×10^6 heat-shocked spores per gram, irradiated and non-irradiated and with 10^4 non-heat-shocked spores per gram, non-irradiated (only 50°F); Run B inoculated with 1×10^2 non-heat-shocked spores per gram, irradiated and non-irradiated and with 10^4 non-heat-shocked spores per gram, non-irradiated (45, 42, and 38°F). The inoculation procedure was the same as described previously.

Procedure for Bacterial Counts and Toxin Assay of Inoculated Skinless Cod Fillets

For Run A of skinless cod fillets inoculated with 10^2 non-heat-shocked spores per gram of type E, Cl. botulinum (Beluga), each of two 100 g samples were blended with 400 ml of phosphate buffer. For clostridial counts, TPG agar with 0.05% sodium thioglycollate, 0.05% ferric citrate, 0.07% sodium sulfite, and 0.01% sodium bicarbonate in Miller-Prickett tubes was used. The tubes were stratified with 0.2% sodium thioglycollate agar. For the aerobic counts, Eugon agar with 0.5% yeast extract and the pour plate technique were used.

For toxin assay two 100 g samples were blended (1 fish to 2 buffer) with gel-phosphate buffer (pH 6.2) and held at 32 to 33°F overnight. The samples were centrifuged in an International refrigerated centrifuge at 8000 rpm for 15 min. A portion of the supernatant was trypsinized (1%) for 45 min at 98.6°F, and the remainder left untrypsinized. Aliquots of 0.5 ml of the trypsinized and non-trypsinized extract were used for intraperitoneal injection into mice, two unprotected and one protected with type E, Cl. botulinum antitoxin.

For pH determinations, a 100 g sample was blended with 100 ml distilled water and the pH read on a Beckman automatic pH meter.

For Run A inoculated with 10^2 non-heat-shocked spores per gram of type E, Cl. botulinum, non-irradiated, and inoculated with 10^4 non-heat-shocked spores per gram of type E, Cl. botulinum, irradiated and non-irradiated, 100 g of each 150 g packaged portion were aseptically removed and blended 1:2 with gel-phosphate buffer (pH 6.2). For the plate counts an aliquot of the 1:2 blend was

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diluted in phosphate buffer (pH 7) to 1:10. The remainder of the blend was held overnight at 33°F prior to processing for toxin assay. TPG agar (as described above) with 0.2% sodium thioglycollate agar as a stratifying layer was used for clostridial counts. Eugon agar with 0.5% yeast extract and the pour plate technique were used for aerobic counts.

The remaining 50 g of cod fillet in the package were blended with an equal weight of boiled distilled water and the pH determined on an automatic Beckman pH meter.

For toxin assay, the 1:2 blend of fish, held overnight at 33°F, was centrifuged in an International refrigerated centrifuge at 8000 rpm for 15 min. A portion of the supernatant was trypsinized (1%) for 45 min at 98.6°F, and the remainder left untrypsinized. Aliquots of 0.5 cc of the trypsinized and non-trypsinized extracts were used for intraperitoneal injection into mice, two unprotected and one protected with type E, Cl. botulinum antitoxin.

For the remaining runs of inoculated cod fillets the 100 g of each package were blended 1:2 with gel-phosphate buffer (pH 6.2). For bacterial counts, an aliquot of the 1:2 blend was diluted in phosphate buffer (pH 7). The remaining blend was held overnight at 33°F prior to processing for toxin assay. The clostridial counts were made in TPG agar (as described previously), using 0.2% sodium thioglycollate agar for stratifying in Miller-Prickett tubes. Eugon agar with 0.5% yeast extract via the streak plate method was used for aerobic counts. (Note: only 100 g portions of fish were inoculated and irradiated in the latter part of the study so as to have enough spores to inoculate sufficient fish to complete the project on time. Therefore, pH tests were suspended at this point.)

For toxin assay the 1:2 blend of fish held overnight at 33°F was centrifuged in an International refrigerated centrifuge at 8000 rpm for 15 min. A portion of the supernatant was trypsinized (1%) for 45 min at 98.6°F and the remainder left untrypsinized. Aliquots of 0.5 cc of the trypsinized and non-trypsinized extracts were used for intraperitoneal injection into mice which had been injected with .4 ml of solution containing 2 mg chloromycetin and .25 mg of terramycin the afternoon prior to the day in which extracts were injected. At the time the mice were injected with the sample, a repeat injection of the antibiotics was made. This was done to obviate non-specific death of the test animals. For each sample (trypsinized or non-trypsinized) two unprotected and one protected (type E antitoxin) mice were used.

RESULTS AND DISCUSSION

Toxicity Tests on Cod Inoculated with Type E, Cl. Botulinum Irradiated or Non-Irradiated and Held at Different Temperatures

The results of toxicity tests on cod inoculated with type E, Cl. botulinum are shown in Tables 18 to 30 inclusive.

It should be noted that in these tests cells of the Beluga strain only of type E, Cl. botulinum were used for inoculation, that inoculation was made with non-heat-shocked cells for the 1×10^2 and 1×10^4 cells per gram inoculation level, and that toxicity tests were made on trypsinized and non-trypsinized portions of a one in three extract of the fillets (1 of fish to 2 of gel-phosphate buffer).

In order to provide a means of comparison, the organoleptic (odor) storage life times of irradiated and non-irradiated cod, as determined by the Fish and Wildlife Service using total rejection by an untrained panel are shown in the following:

Storage Life of Cod

Storage Temp. (°F)	Storage Life Time (Days) at Dose Level (Krad)		
	none	100	200
50	5	7	10
46	5	10	15
42	7	18	25
33	10	30	40

A summary of the results of toxicity tests with type E, Cl. botulinum inoculated cod portions (taken from the data in Table 18 to 30 inclusive) is shown below:

Cod

Storage Temp. (°F)	Run A			Run B		
	Toxicity Time (Days) at Dose Level (Krad)			Toxicity Time (Days) at Dose Level (Krad)		
	none	100	200	none	100	200
	<u>1 x 10² Inoculum</u>					
50	--	>16	>16	18	18	14
46	--	>20	>20	--	--	--
45	10	--	--	>24	36	22
42	14	>26	>36	>24	>21	>24
38	16	>30	>42	>30	>50	>50

Cod (continued)

Storage Temp. (°F)	Run A			Run B		
	Toxicity Time (Days) at Dose Level (Krad)			Toxicity Time (Days) at Dose Level (Krad)		
	none	100	200	none	100	200
1 x 10 ⁴ Inoculum						
50	--	4*	9*	18		
46	--	--	--	--		
45	14	18	11*	10		
42	14	>32	27	>24		
38	15	>42	>42	>31		
1 x 10 ⁶ Inoculum						
50	10	8	14			
45	14	12	17			
42	12	17	>24			
38	>30	>34	>34			

It can be seen that the only questionable results regarding the safety of radiation-pasteurized cod were obtained at 50°F (four days for 100 krad and nine days for 200 krad-treated) and at 45°F (eleven days for the 200 krad-treated). These results (marked with an asterisk in the above table) indicate shorter times for toxicity than the time judged to be the organoleptic storage life for such products by the Fish and Wildlife Service untrained panel.

It would appear that such results need not be the cause for great concern due to the entire unlikelihood that fresh fish that would ever be subjected to radiation-pasteurization treatment would under any circumstance contain 1 x 10⁴ cells per gram of type E, Cl. botulinum or concentrations remotely approaching this level.

It is obvious that the use of non-heat-shocked cells (as in the 1×10^2 and 1×10^4 cells per gram levels of inoculum) has in some way biased the results of toxicity of inoculated, irradiated cod as compared to the use of heat-shocked spores as inocula. For instance, the use of inocula of non-heat-shocked cells at the 1×10^4 per gram level in two out of two tests at 50°F and in one out of two tests at 45°F provided toxic fillets at periods sooner than those obtained by using heat-shocked spores at a level of 1×10^6 per gram. Such results would seem to be anomalous.

It can be seen from Tables 18 to 30 inclusive that in some instances in which toxin was obtained, both in non-irradiated and irradiated samples, there was some kind of proto-toxin carryover when non-heat-shocked cells were used. This is indicated by the fact that in these cases there was no increase in the clostridial count when toxin was present.

Whether there is a possibility that the use of non-heat-shocked cells in some way enhances the growth of type E, Cl. botulinum in inoculated areas through lysis of whatever vegetative cells are present and utilization of this material by spores for purposes of outgrowth may also be questioned. It should be realized, for instance, that in order to add 1×10^4 cells per gram to pieces of fish by inoculation in a limited number of areas with a hypodermic needle it is necessary to add more than 1×10^6 cells per gram to any particular area where the inoculation is made.

It is considered that the method of using non-heat-shocked cells for inoculation to determine toxicity development in irradiated and non-irradiated samples should be re-evaluated.

TABLE 18. Test Results for Skinless Cod Fillets Inoculated with 10^2 Non-Heat Shocked Spores of Type E, Cl. botulinum (Beluga) and Non-Irradiated (Run A)

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity		pH of	
		of Samples		of Samples		of Samples		Samples	
		1	2	1	2	1	2	1	2
45°F	2	1×10^1	$.5 \times 10^1$	1.7×10^6	7.8×10^5	-	-	7.1	7.0
	4	1.5×10^1	$< 10^1$	8.3×10^7	5.7×10^7	-	-	7.1	7.0-7.1
	6	$< 10^1$	$< 10^1$	3.2×10^8	1.3×10^8	-	-	7.3	7.2-7.3
	10	$< 10^1$	$< 10^1$	3.0×10^8	3.6×10^8	+	-	7.1	7.1-7.2
	14	$< 10^1$	$< 10^1$	2.3×10^8	3.8×10^8	+	+	7.5	7.4-7.5
	18	$< 10^1$	$< 10^1$	4.7×10^8	4.9×10^8	+	+	7.8-7.9	7.8
	22	$< 10^1$	$< 10^1$	6.6×10^8	6.4×10^8	+	+	7.5-7.6	7.6-7.7
42°F	3	$< 10^1$	$.5 \times 10^1$	1.8×10^7	3.8×10^7	-	-	7.0-7.1	7.0
	5	1×10^1	2×10^1	1.6×10^7	2.8×10^7	-	-	7.1-7.2	7.2
	7	5×10^1	1.1×10^2	6.1×10^7	9.9×10^7	-	-	6.9-7.0	7.1-7.2
	11	$< 10^1$	$< 10^1$	7.5×10^7	6.2×10^7	-	-	7.2	7.1-7.2
	14	$< 10^1$	$< 10^1$	1.4×10^8	7.3×10^7	+	+	7.3	7.2
	18	$< 10^1$	1×10^1	3.8×10^8	1.4×10^8	+	-	7.4-7.5	7.4
	25	$< 10^1$	$< 10^1$	5.2×10^8	5.3×10^8	+	*	8.1	7.9
	35	$< 10^1$	$< 10^1$	6.1×10^8	6.9×10^8	+	-	7.8	7.8-7.9

TABLE 18. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity		pH of	
		of Samples		of Samples		of Samples		Samples	
		1	2	1	2	1	2	1	2
38°F	3	2.0×10^2	3.3×10^2	6.5×10^6	4.4×10^7	-	-	7.1	7.2
	5	9×10^1	2×10^2	1.3×10^7	1.4×10^7	-	-	7.1	7.1-7.2
	11	$< 10^1$	$< 10^1$	7.6×10^7	1.9×10^8	-	-	7.0-7.1	7.1
	16	2×10^2	2×10^1	2.0×10^8	7.6×10^7	+	-	7.1	7.0-7.1
	20	$< 10^1$	$< 10^1$	1.4×10^8	2.9×10^8	-	+	7.7	7.1
	25	$< 10^1$	$< 10^1$	5.3×10^8	3.7×10^8	+	+	7.7	7.6
	35	$< 10^1$	$< 10^1$	6.2×10^8	9.0×10^8	-	-	7.8	7.9

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

* = non-specific

TABLE 19. Test Results for Skinless Cod Fillets Inoculated with 10^2 Non-Heat Shocked Spores of Type E, Cl. Botulinum (Beluga) and Irradiated at 100 Krad (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity		pH of	
		<u>of Samples</u>		<u>of Samples</u>		<u>cf Samples</u>		<u>Samples</u>	
		1	2	1	2	1	2	1	2
50°F	2	9×10^1	5×10^1	4.3×10^3	1.1×10^4	-	-	6.5	
	6	$< 10^1$	$< 10^1$	3×10^7	1.7×10^7	-	-	6.8	
	10	$< 10^1$	$< 10^1$	2.3×10^7	2.8×10^7	-	-	6.8	
	16	1.8×10^3	8.0×10^2	2.6×10^8	6.3×10^7	-	-	6.7	
46°F	3	5×10^1	2×10^1	3.4×10^3	8.0×10^3	-	-	6.5	
	7	$.5 \times 10^1$	$< 10^1$	1.1×10^7	2.0×10^7	-	-	6.4	
	14	$< 10^1$	$< 10^1$	1.5×10^8	8.6×10^7	-	-	6.5	
	20	2.5×10^4	$< 10^1$	1.9×10^8	1.7×10^8	-	-	6.7	
42°F	3	2×10^1	4×10^1	5×10^2	9×10^2	-	-	6.5	
	8	1×10^1	1×10^1	4.5×10^5	2.8×10^6	-	-	6.7	
	16	$< 10^1$	$< 10^1$	5.6×10^7	5.1×10^7	-	-	6.6	
	26	$< 10^1$	$< 10^1$	3.5×10^8	8.0×10^8	-	-	6.8	

TABLE 19. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity		pH of	
		<u>of Samples</u>		<u>of Samples</u>		<u>of Samples</u>		<u>Samples</u>	
		1	2	1	2	1	2	1	2
38°F	6	< 10 ¹	< 10 ¹	2.0 x 10 ²	1.6 x 10 ⁴	-	-	6.4	
	10	< 10 ¹	< 10 ¹	3.1 x 10 ⁵	5.8 x 10 ⁵	-	-	6.4	
	20	< 10 ¹	< 10 ¹	1.0 x 10 ⁷	4.2 x 10 ⁷	-	-	6.5	
	30	< 10 ¹	< 10 ¹	2.7 x 10 ⁸	4.8 x 10 ⁸	-	-	7.0	

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 20. Test Results for Skinless Cod Fillets Inoculated with 10^2 Non-Heat Shocked Spores of Type E, Cl. botulinum (Beluga) and Irradiated at 200 Krad (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples		pH of Samples	
		1	2	1	2	1	2	1	2
50°F	5	4×10^2	contaminated	1.7×10^6	contaminated	-	-	6.7	
	8	$< 10^1$	$< 10^1$	1.1×10^8	9.9×10^7	-	-	6.9	
	16	$< 10^1$	$< 10^1$	6.6×10^7	8.1×10^7	-	-	6.9	
46°F	6	$< 10^1$	$< 10^1$	1.3×10^6	4.7×10^6	-	-	6.6	
	9	$< 10^1$	$< 10^1$	5.1×10^6	1.9×10^5	-	-	6.6	
	20	$< 10^1$	$< 10^1$	1.2×10^8	8.5×10^7	-	-	6.8	
42°F	7	$< 10^1$	$.5 \times 10^1$	6.3×10^4	3.5×10^4	-	-	6.7	
	12	$< 10^1$	$< 10^1$	8.8×10^7	1.6×10^6	-	-	6.5	
	24	$< 10^1$	$< 10^1$	4.3×10^8	8.0×10^6	-	-	6.5	
	36	$< 10^1$	$< 10^1$	1.6×10^7	1.4×10^7	-	-	6.5	
38°F	7	$< 10^1$	$.5 \times 10^1$	7.7×10^5	1.1×10^6	-	-	6.7	
	14	$< 10^1$	$< 10^1$	7.3×10^5	3.0×10^5	-	-	6.5	
	28	$< 10^1$	$< 10^1$	1.5×10^8	5.2×10^7	-	-	6.5	
	42	$< 10^1$	$< 10^1$	3×10^8	1.4×10^6	-	-	6.5	

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 21. Test Results for Skinless Cod Fillets Inoculated with 10^4 Non-Heat Shocked Spores of Type E, Cl. botulinum (Beluga) and Non-Irradiated (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples		pH of Samples	
		1	2	1	2	1	2	1	2
45°F	2	5×10^2	1.4×10^3	7.5×10^6	7.6×10^6	-	-	7.1	7.2
	4	5.1×10^2	5.2×10^2	1.5×10^7	4.3×10^7	-	-	7.0-7.1	7.1
	6	$< 10^1$	$< 10^1$	3.4×10^8	2.7×10^8	-	-	7.5-7.6	7.3-7.4
	10	$< 10^1$	$< 10^1$	1.8×10^8	3.7×10^8	-	-	7.4-7.5	7.2-7.3
	14	$< 10^1$	$< 10^1$	3.6×10^8	4.6×10^7	*	+	7.4-7.5	7.4
	18	$< 10^1$	$< 10^1$	4.2×10^8	7.6×10^8	+	+	7.5	7.5
	22	$< 10^1$	$< 10^1$	4.5×10^8	6.1×10^8	+	+	7.9	7.9-8.0
42°F	3	2×10^1	1×10^1	3.8×10^7	4.4×10^7	-	-	7.0-7.1	7.0-7.1
	5	1×10^1	7×10^1	1.9×10^7	2.8×10^7	-	-	7.0-7.1	7.2
	7	3×10^1	1×10^1	9.6×10^7	9.6×10^7	-	-	7.2-7.3	7.1
	11	7×10^1	1×10^1	1.7×10^8	2.2×10^8	-	-	7.3-7.4	7.2
	14	$< 10^1$	$< 10^1$	3.6×10^8	7.6×10^8	*	+	7.4	7.2
	18	$< 10^1$	$< 10^1$	6.3×10^8	4.8×10^8	+	+	7.3	7.6
	25	$< 10^1$	$< 10^1$	7.4×10^8	7.4×10^8	+	+	8.0	8.0-8.1
	35	$< 10^1$	$< 10^1$	6.9×10^8	7.4×10^8	+	-	7.7	7.9

TABLE 21. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples		pH of Samples	
		1	2	1	2	1	2	1	2
38°F	3	2.4×10^3	2.1×10^3	4.0×10^6	4.3×10^7	-	-	6.9-7.0	7.1
	5	2.1×10^3	7.0×10^2	2.7×10^6	9.7×10^6	-	-	7.2	7.2-7.3
	11	6×10^2	3×10^2	1.1×10^8	1.0×10^8	-	-	7.3	7.2
	16	$> 10^2$	$< 10^1$	4.2×10^8	1.6×10^8	+	+	7.5	7.2
	20	$< 10^1$	$< 10^1$	8.3×10^7	5.5×10^7	+	-	7.5	7.7-7.8
	25	$< 10^1$	$< 10^1$	3.3×10^8	3.6×10^8	-	-	7.6	8.0-8.1
	35	$< 10^1$	$< 10^1$	8.0×10^8	6.6×10^8	+	-	7.9-8.0	7.4

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

* = non-specific

TABLE 22. Test Results for Skinless Cod Fillets Inoculated with 10^4 Non-Heat Shocked Spores of Type E, C_l. botulinum (Beluga) and Irradiated at 100 Krad.

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples		pH of Samples	
		1	2	1	2	1	2	1	2
50°F	4	3×10^4	2.0×10^4	3.5×10^8	3.8×10^8	+	-	not done	not done
	9	7.2×10^5	$>1 \times 10^6$	6.0×10^8	3.4×10^8	+	+	not done	not done
	12	8.9×10^6	5×10^5	5.2×10^8	5.2×10^8	+	+	not done	not done
45°F	2	8×10^2	6×10^2	5.3×10^5	8.8×10^5	-	-	6.9-7.0	6.7-6.8
	5	5×10^1	2×10^1	2.6×10^7	1.2×10^7	-	-	7.0-7.1	7.2
	8	$< 10^1$	3.5×10^3	9.3×10^7	2.8×10^8	-	-	7.0	7.0-7.1
	11	$< 10^1$	$< 10^1$	2.9×10^8	2.5×10^8	-	-	7.2-7.3	6.9
	14	$< 10^1$	$< 10^1$	5.0×10^8	5.5×10^8	-	-	7.2	7.1
	18	6×10^3	1.1×10^5	9.5×10^8	3.0×10^8	+	-	7.2	7.2-7.3
	23	1.4×10^5	1.2×10^5	7.4×10^7	5.6×10^8	-	+	7.2	7.3
42°F	28	2.4×10^6	3.4×10^5	2.0×10^8	1.0×10^8	+	+	7.4	7.5-7.6
	4	1.0×10^3	9×10^2	1.1×10^7	1.1×10^7	-	-	7.0	7.0
	7	4×10^2	between 1×10^2 & 1×10^3	2.5×10^7	7.6×10^7	-	-	7.3	7.2

TABLE 22. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples		pH of Samples	
		1	2	1	2	1	2	1	2
42°F	10	< 10 ¹	< 10 ¹	2.3 x 10 ⁸	9.8 x 10 ⁷	-	-	6.8-6.9	6.9-7.0
	15	< 10 ¹	< 10 ¹	3.1 x 10 ⁸	2.2 x 10 ⁸	-	-	7.1	6.9-7.0
	20	< 10 ¹	< 10 ¹	2.6 x 10 ⁸	6.1 x 10 ⁸	-	-	7.3	7.3-7.4
	27	< 10 ¹	< 10 ¹	2.4 x 10 ⁸	2.5 x 10 ⁸	-	-	7.7	7.4-7.5
	32	2 x 10 ⁴	1 x 10 ⁴	1.5 x 10 ⁸	2.4 x 10 ⁸	-	-	not done	not done
38°F	5	1.2 x 10 ³	6.5 x 10 ²	3.4 x 10 ⁶	2.7 x 10 ⁶	-	-	7.0-7.1	7.0
	10	< 10 ¹	< 10 ¹	8.2 x 10 ⁷	6.3 x 10 ⁷	-	-	7.0	6.7
	15	< 10 ¹	< 10 ¹	1.9 x 10 ⁸	1.8 x 10 ⁸	-	-	7.1	6.8-6.9
	20	< 10 ¹	< 10 ¹	4.6 x 10 ⁸	4.4 x 10 ⁸	-	-	7.2	7.3
	25	< 10 ¹	< 10 ¹	1.3 x 10 ⁸	3.3 x 10 ⁸	-	-	7.2-7.3	7.1
	32	.5 x 10 ¹	.5 x 10 ¹	3.8 x 10 ⁸	2.3 x 10 ⁸	-	-	not done	not done
	42	< 10 ¹	< 10 ¹	5.3 x 10 ⁸	3.5 x 10 ⁸	-	-	7.3	7.3

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 23. Test Results for Skinless Cod Fillets Inoculated with 10^4 Non-Heat Shocked Spores of Type E, *Cl. botulinum* (Beluga) and Irradiated at 200 Krad.

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples		pH of Samples	
		1	2	1	2	1	2	1	2
50°F	4	1×10^3	2.1×10^4	1.1×10^8	1.7×10^8	-	-	not done	not done
	9	1.8×10^6	1.9×10^6	5.2×10^8	2.2×10^8	+	+	not done	not done
	15	$>1 \times 10^6$	$>1 \times 10^6$	8.9×10^8	6.4×10^8	+	+	not done	not done
45°F	2	1.1×10^2	2.0×10^2	1.9×10^5	2.9×10^5	-	-	6.8-6.9	6.8-6.9
	5	4×10^1	3.3×10^1	1.7×10^7	1.9×10^7	-	-	6.8	7.1
	8	$< 10^1$	5×10^2	7.0×10^7	1.1×10^8	-	-	7.2	7.2
	11	$< 10^1$	5×10^3	3.8×10^8	1.6×10^8	-	+	6.9	7.0
	14	$>1 \times 10^3$	$>1 \times 10^3$	7.0×10^8	3.7×10^8	-	-	7.1	7.0-7.1
	18	7.4×10^4	2.2×10^5	9.1×10^8	5.5×10^8	-	-	7.0	7.0
	23	3.3×10^6	2.2×10^6	4.4×10^8	2.1×10^8	+	-	7.5	7.2
	28	3.2×10^6	1.4×10^6	2.5×10^8	2.6×10^8	+	+	7.5	7.5
42°F	4	9×10^1	8×10^1	1.1×10^7	7.0×10^6	-	-	7.0-7.1	7.1
	7	$< 10^1$	1×10^1	8.9×10^7	3.4×10^6	-	-	7.4	7.1
	10	5.4×10^2	8×10^1	1.7×10^8	1.8×10^8	-	-	7.0	7.0

TABLE 23. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity		pH of	
		of Samples		of Samples		of Samples		Samples	
		1	2	1	2	1	2	1	2
42°F	15	1.1×10^3	2.1×10^4	2.1×10^8	3.1×10^8	-	-	7.1	6.9
	20	8×10^5	$>1 \times 10^5$	3.3×10^8	3.5×10^8	-	-	7.3	7.5-7.6
	27	$>1 \times 10^5$	$>1 \times 10^5$	5.2×10^8	2.5×10^8	+	-	7.5	7.5-7.6
	32	5×10^6	6×10^5	1.5×10^8	2.5×10^8	+	+	not done	not done
38°F	5	1.1×10^2	2.2×10^2	5.0×10^5	1.0×10^6	-	-	7.1	6.9
	10	2×10^1	1×10^2	6.7×10^7	7.5×10^7	-	-	7.0	6.9
	15	$< 10^1$	2×10^1	1.8×10^8	1.2×10^8	-	-	7.2	7.2
	20	5×10^1	$< 10^1$	2.5×10^8	1.5×10^8	-	-	7.2	7.3
	27	$< 10^1$	$.5 \times 10^1$	4.2×10^7	1.3×10^8	-	-	7.3-7.4	7.1-7.2
	32	1×10^2	$.5 \times 10^1$	6.1×10^8	6.6×10^8	-	-	not done	not done
	42	$< 10^1$	$< 10^1$	2.0×10^8	3.3×10^8	-	-	7.3-7.4	7.2-7.3

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 24. Test Results for Skinless Cod Fillets Inoculated with 10^2 Non-Heat Shocked Spores of Type E, Cl. botulinum (Beluga) and Irradiated at 200 Krad (Run B).

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples		of Samples			
		1	2	1	2	1	2
50°F	4	5×10^1	2×10^2	8.0×10^6	7.5×10^7	-	-
	8	between 1×10^1 _{&} 1×10^2	$< 10^1$	2.0×10^8	6.3×10^7	-	-
	14	2×10^1	$< 10^1$	9.9×10^8	7.9×10^8	+	-
	20	6×10^5	lost	5.4×10^8	lost	+	lost
	24	4.2×10^6	5×10^5	7.6×10^8	6.4×10^8	+	+
45°F	5	1.5×10^2	5×10^1	7.1×10^7	6.7×10^7	-	-
	11	2×10^2	8×10^2	6.9×10^8	4.0×10^8	-	-
	16	between 1×10^3 _{&} 1×10^4	1.5×10^4	9.1×10^8	5.1×10^8	-	-
	22	5×10^4	1×10^4	5.2×10^8	3.9×10^9	-	+
	33	1.3×10^6	1.8×10^6	6.1×10^8	7.0×10^8	-	+
42°F	18	2×10^1	1×10^1	4.3×10^8	4.8×10^8	-	-
	19	$< 10^1$	$< 10^1$	2.6×10^8	4.4×10^8	-	-

TABLE 24. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples	
		1	2	1	2	1	2
42°F	24	< 10 ¹	< 10 ¹	5.2 x 10 ⁸	2.8 x 10 ⁸	-	-
	45	< 10 ¹	< 10 ¹	4.4 x 10 ⁸	5.2 x 10 ⁸	-	-
38°F	18	< 10 ¹	< 10 ¹	4.0 x 10 ⁸	5.7 x 10 ⁸	-	-
	25	< 10 ¹	< 10 ¹	2.3 x 10 ⁸	4.4 x 10 ⁸	-	-
	34	< 10 ¹	< 10 ¹	6.9 x 10 ⁸	2.2 x 10 ⁸	-	-
	50	< 10 ¹	< 10 ¹	4.6 x 10 ⁸	4.3 x 10 ⁸	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 25. Test Results for Skinless Cod Fillets Inoculated with 10^2 Non-Heat Shocked Spores of Type E, Cl. botulinum (Beluga) and Irradiated at 100 Krad (Run B).

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		<u>of Samples</u>		<u>of Samples</u>		<u>of Samples</u>	
		1	2	1	2	1	2
50°F	4	5×10^1	2×10^1	5.2×10^7	1.1×10^8	-	-
	8	$< 10^1$	1×10^4	5.2×10^8	3.5×10^8	-	-
	12	1.8×10^5	3.3×10^5	6.4×10^8	8.8×10^8	*	*
	18	$< 10^2$	not done	8.9×10^8	not done	+	not done
	24	8×10^5	2×10^5	1.1×10^9	7.2×10^8	+	+
45°F	5	$.5 \times 10^1$	5×10^1	1.1×10^8	8.0×10^7	-	-
	9	$< 10^1$	$< 10^1$	4.7×10^8	6.8×10^8	-	-
	12	$< 10^1$	$< 10^1$	4.0×10^8	6.3×10^8	-	-
	18	8×10^4	1×10^3	6.4×10^8	6.5×10^8	-	-
	25	6×10^5	2.5×10^5	5.7×10^8	5.9×10^8	-	-
	36	5×10^4	6×10^5	7.1×10^8	1.1×10^9	-	+
42°F	11	$< 10^1$	$< 10^1$	3.8×10^8	7.2×10^8	-	-
	21	$< 10^1$	$< 10^1$	5.0×10^8	1.1×10^9	-	-
	22	$< 10^1$	$< 10^1$	6.7×10^8	2.9×10^8	-	-
	34	$< 10^1$	$< 10^1$	8.3×10^8	7.7×10^8	-	-
	42	$< 10^1$	$< 10^1$	8.1×10^8	9.0×10^8	-	-

TABLE 25. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		<u>of Samples</u>	<u>of Samples</u>	<u>of Samples</u>	<u>of Samples</u>	<u>of Samples</u>	<u>of Samples</u>
		1	2	1	2	1	2
38°F	18	< 10 ¹	< 10 ¹	5.4 x 10 ⁸	6.1 x 10 ⁸	-	-
	34	< 10 ¹	< 10 ¹	5.1 x 10 ⁸	3.8 x 10 ⁸	-	-
	50	< 10 ¹	< 10 ¹	7.2 x 10 ⁸	8.4 x 10 ⁸	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

* = non-specific

TABLE 26. Test Results for Skinless Cod Fillets Inoculated with 10^2 Non-Heat Shocked Spores of Type E, C. botulinum (Beluga) and Non-Irradiated (Run B).

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples	
		1	2	1	2	1	2
50°F	3	< 10^1	< 10^1	5.6×10^8	3.4×10^8	-	-
	6	< 10^1	< 10^1	9.5×10^8	8.2×10^8	-	-
	9	< 10^1	< 10^1	1.1×10^9	1.2×10^9	-	-
	12	< 10^1	< 10^1	1.5×10^9	2.9×10^9	-	-
	18	2×10^5	1.5×10^5	1.2×10^9	1.1×10^9	+	+
	24	2.3×10^5	8×10^5	1.0×10^9	6.1×10^8	+	+
45°F	3	< 10^1	< 10^1	4.8×10^8	3.3×10^8	-	-
	6	< 10^1	< 10^1	7.1×10^8	8.0×10^8	-	-
	9	< 10^1	< 10^1	1.0×10^9	1.3×10^9	-	-
	12	< 10^1	< 10^1	$>1 \times 10^8$	1.1×10^9	-	-
	18	< 10^1	< 10^1	1.7×10^9	6.8×10^8	-	-
	24	< 10^1	< 10^1	9.3×10^8	7.5×10^8	-	-
42°F	4	< 10^1	< 10^1	2.5×10^8	1.8×10^8	-	-
	8	< 10^1	< 10^1	6.9×10^8	1.2×10^9	-	-
	13	< 10^1	< 10^1	9.0×10^8	7.8×10^8	-	-
	19	< 10^1	< 10^1	1.1×10^9	9.4×10^8	-	-
	24	< 10^1	< 10^1	4.3×10^8	6.6×10^8	-	-

TABLE 26. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples		of Samples			
		1	2	1	2	1	2
38°F	5	< 10 ¹	< 10 ¹	1.0 x 10 ⁸	1.6 x 10 ⁸	-	-
	10	< 10 ¹	< 10 ¹	9.7 x 10 ⁸	6.7 x 10 ⁸	-	-
	16	< 10 ¹	< 10 ¹	5.4 x 10 ⁸	6.3 x 10 ⁸	-	-
	20	< 10 ¹	< 10 ¹	2.3 x 10 ⁸	5.1 x 10 ⁸	-	-
	30	< 10 ¹	< 10 ¹	7.9 x 10 ⁸	5.3 x 10 ⁸	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 27. Test Results for Skinless Cod Fillets Inoculated with 10^4 Non-Heat Shocked Spores of Type E, Cl. botulinum (Beluga) and Non-Irradiated (Run B).

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples		of Samples			
		1	2	1	2	1	2
50°F	3	$< 10^1$	$< 10^1$	3.6×10^8	2.6×10^8	-	-
	6	$< 10^1$	$< 10^1$	8.1×10^8	1.9×10^9	-	-
	9	7.9×10^5	3×10^4	1.2×10^9	1.2×10^9	-	-
	12	5.3×10^4	1×10^4	1.2×10^9	1.0×10^9	-	-
	18	1×10^5	3×10^4	1.7×10^9	7×10^8	-	+
	23	1.1×10^5	4×10^5	1.2×10^9	1.6×10^9	+	-
45°F*	2	$.5 \times 10^4$	$.5 \times 10^4$	9.7×10^6	4.7×10^6	-	-
	4	3×10^4	1.4×10^3	3.6×10^7	1.2×10^7	-	-
	6	$< 10^1$	$< 10^1$	3.5×10^8	5.7×10^8	-	-
	10	$< 10^1$	$< 10^1$	3.5×10^8	5.7×10^8	-	+
	14	$< 10^1$	between 1×10^4 & 1×10^5	2.6×10^8	4.3×10^8	+	+
	19	1.0×10^5	$>1 \times 10^4$	5.8×10^8	4.5×10^8	-	-
42°F	4	$< 10^1$	$< 10^1$	1.9×10^8	1.8×10^8	-	-
	8	$< 10^1$	$< 10^1$	8.7×10^8	1.5×10^9	-	-
	12	$< 10^1$	$< 10^1$	1.6×10^9	7.8×10^8	-	-

TABLE 27. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples	
		1	2	1	2	1	2
42°F	12	8×10^2	1×10^3	4.5×10^8	7.6×10^8	-	-
	19	3×10^2	$< 10^1$	7.5×10^8	6.8×10^8	-	-
	24	$< 10^1$	1×10^2	5.9×10^8	8.3×10^8	-	-
38°F	5	6×10^2	1×10^1	3.0×10^8	3.6×10^8	-	-
	10	$< 10^1$	$< 10^1$	1.1×10^9	1.1×10^9	-	-
	15	$< 10^1$	$< 10^1$	8.2×10^8	7.6×10^8	-	-
	20	$< 10^1$	$< 10^1$	8.7×10^8	6.9×10^8	-	-
	31	$< 10^1$	$< 10^1$	6.4×10^8	7.6×10^8	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

*This repeat run was done with a different spore crop than 50, 42, and 38°F and the toxin assay was preformed without antibiotics.

TABLE 28. Test Results for Skinless Cod Fillets Inoculated with 10^6 Heat Shocked Spores of Type E, Cl. botulinum (Beluga) and Non-Irradiated (Run A).

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples		of Samples			
		1	2	1	2	1	2
50°F	4	6.0×10^3	7.0×10^3	5.6×10^8	6.4×10^8	-	-
	6	1.0×10^5	6×10^5	8.8×10^8	5.5×10^8	-	-
	10	3.1×10^5	5.1×10^5	5.4×10^8	4.3×10^8	+	+
	13	7.0×10^6	2×10^5	1.8×10^9	1.5×10^9	+	+
	18	5×10^5	1.2×10^6	1.1×10^9	2.8×10^9	+	+
45°F	2	5×10^3	6×10^3	6.1×10^8	7.2×10^8	-	-
	4	1×10^1	4×10^1	6.7×10^8	2.8×10^8	-	-
	6	6×10^3	6×10^3	4.6×10^8	4.0×10^8	-	-
	10	1.4×10^2	$< 10^1$	5.0×10^8	5.9×10^8	-	-
	14	$> 1 \times 10^3$	$< 10^1$	3.8×10^8	3.7×10^8	+	+
	19	5×10^5	between	3.0×10^8	2.5×10^8	-	-
			1×10^4 & 1×10^5				
	22	between	between	2.5×10^8	6.3×10^8	+	-
		1×10^4 & 1×10^5	1×10^4 & 1×10^5				

TABLE 28. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples	
		1	2	1	2	1	2
42°F	4	1.2×10^4	5.5×10^3	2.2×10^8	1.7×10^8	-	-
	8	5×10^3	5×10^4	2.2×10^8	2.8×10^8	-	-
	12	8×10^2	1×10^2	7.6×10^8	7.5×10^8	-	+
	18	1×10^3	$< 10^2$	9.6×10^8	6.2×10^8	-	-
	24	$< 10^1$	$< 10^1$			-	-
38°F	5	1.5×10^4	4×10^4	4.1×10^7	1.1×10^8	-	-
	16	1×10^1	1×10^4	1.7×10^9	6.3×10^8	-	-
	20	2.0×10^4	7×10^4	1.6×10^9	6.1×10^8	-	-
	30	1×10^4	1×10^3	2.2×10^8	5.8×10^8	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 29. Test Results for Skinless Cod Fillets Inoculated with 10^6 Heat Shocked Spores of Type E, Cl. botulinum (Beluga) and Irradiated at 100 Krad.

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		of Samples		of Samples			
		1	2	1	2	1	2
50°F	5	1×10^1	2.5×10^2	1.1×10^8	2.9×10^8	-	-
	8	2.0×10^6	1.5×10^6	4.8×10^8	6.4×10^8	+	+
	12	$>1 \times 10^6$	$>1 \times 10^6$	5.0×10^8	4.9×10^8	+	+
45°F	6	1×10^3	1×10^4	7.8×10^7	1.3×10^8	-	-
	12	2.7×10^6	4×10^5	5.0×10^8	8.2×10^8	+	+
42°F	5	5×10^3	4×10^3	9.9×10^7	1.2×10^8	-	-
	11	1×10^1	1×10^4	5.8×10^8	5.3×10^8	-	-
	17	4×10^3	8×10^4	5.6×10^8	6.5×10^8	-	+
	22	8×10^4	between 1×10^2 & 1×10^3	6.9×10^8	8.4×10^8	+	-
	35	1×10^1	10^1	7.1×10^8	5.0×10^8	-	+
	42	4×10^4	10^1	4.8×10^8	8.7×10^8	-	-
38°F	8	4×10^1	1×10^1	9.3×10^7	1.4×10^8	-	-
	18	5×10^2	5×10^2	4.6×10^8	5.6×10^8	-	-

TABLE 29. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples	
		1	2	1	2	1	2
38°F	25	< 10 ²	< 10 ²	6.5 x 10 ⁸	1.1 x 10 ⁹	-	-
	34	< 10 ¹	< 10 ¹	8.3 x 10 ⁸	8.1 x 10 ⁸	-	-
	52	< 10 ¹	< 10 ¹	5.6 x 10 ⁸	7.1 x 10 ⁸	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

TABLE 30. Test Results for Skinless Cod Fillets Inoculated with 10^6 Heat Shocked Spores of Type E, Cl. botulinum (Beluga) and Irradiated at 200 Krad.

Storage Temp.	Storage Time (Days)	Clostridial Counts of Samples		Aerobic Counts of Samples		Toxicity of Samples	
		1	2	1	2	1	2
50°F	4	1×10^5	1×10^3	6.6×10^7	9.9×10^7	-	-
	8	2×10^5	2.0×10^2	3.2×10^8	7.7×10^8	-	-
	14	6×10^6	9×10^6	4.9×10^8	6.3×10^8	+	+
45°F	6	1×10^2	1×10^2	4.8×10^8	1.2×10^7	-	-
	11	$< 10^1$	3.1×10^2	2.3×10^8	3.3×10^8	-	-
	17	1×10^5	8×10^4	9.5×10^8	2.4×10^8	+	+
	23	3.5×10^6	1.0×10^5	5.7×10^8	1.1×10^9	-	-
42°F	7	4×10^2	5×10^2	4.5×10^7	2.7×10^7	-	-
	12	1×10^3	1×10^3	6.0×10^8	4.5×10^8	-	-
	18	1×10^3	8×10^2	3.7×10^8	4.2×10^8	-	-
	24	5×10^1	1×10^2	2.1×10^8	6.8×10^8	-	-
	46	5×10^2	6×10^2	3.9×10^8	4.4×10^8	+	-
38°F	8	4×10^1	2×10^1	7.3×10^7	1.1×10^8	-	-
	18	1×10^1	3.5×10^1	2.7×10^8	4.3×10^8	-	-

TABLE 30. (continued)

Storage Temp.	Storage Time (Days)	Clostridial Counts		Aerobic Counts		Toxicity of Samples	
		<u>of Samples</u>		<u>of Samples</u>		<u>of Samples</u>	
		1	2	1	2	1	2
38°F	25	< 10 ²	< 10 ²	1.7 x 10 ⁸	8.1 x 10 ⁸	-	-
	34	< 10 ¹	< 10 ¹	1.3 x 10 ⁹	7.8 x 10 ⁸	-	-
	52	< 10 ¹	< 10 ¹	9.7 x 10 ⁸	7.1 x 10 ⁸	-	-

+ indicates that the sample was toxic in the 1:10 dilution.

- indicates that the sample was not toxic in the 1:10 dilution.

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