RECEIVED BY DTIC NOV 19 1968

COO-1183-27

CONF-681028--1

MASTER

Inoculated Pack Studies on Clostridium
botulinum type E in Unirradiated and Irradiated Haddock.

W. P. Segner and C. F. Schmidt

Metal Division Research and Development

CONTINENTAL CAN COMPANY, INC.

Chicago, Illinois 60620

For presentation at the UJNR Conference on Toxic
Microorganisms, Honolulu, Hawaii, October 7-10, 1968.

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED
Pat

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

ABSTRACT

Inoculated packs were initiated to assess the public health aspect of Clostridium botulinum type E in radurized and unirradiated haddock. Estimates of the maximal expected product storage life were determined by an untrained consumer-type panel. Values of storage life are designated as X and represent the time for unanimous product rejection by this panel. Triplicate samples per variable were inoculated with 10^6 , 10^4 , or 10^2 of the Beluga strain/g. Samples were given 0.1 and 0.2 Mrad doses and incubated along with inoculated unirradiated controls at intervals from 50 F (10 C) to 40 F (4.4 C). Toxin assays were conducted at fractions and multiples of X. At 40 and 42 F incubation, control and irradiated samples inoculated with 10^6 and 10^4 levels showed no toxin up to 2X. At 46 F incubation, samples inoculated with 10^6 spores and given 0.1 Mrad irradiation showed toxin at X, but not earlier; controls remained nontoxic through 2X. Control and 0.1 Mrad samples with a 10^4 inoculum showed no toxin development up to 2X. With 0.2 Mrad irradiation, toxin was detected at 1/2X for both inoculum levels. At

50 F, control and 0.1 Mrad irradiated samples were nontoxic up to X with 10^4 and 10^2 inoculum levels. However, 0.2 Mrad samples showed detectable toxin at 1/2X and 3/4X with 10^4 and 10^2 inoculum levels. The results with 0.1 Mrad appear encouraging, but the data with 0.2 Mrad irradiation are less favorable.

The commercial distribution of fresh seafood on the mainland of the United States is limited primarily to those coastal areas where seafood is caught, because of its extremely high perishability. The US Atomic Energy Commission has proposed the possible exploitation of low doses of ionizing radiation, or so-called radurization doses (4), to extend the refrigerated storage life of certain marine food products.

Radurization doses of 0.1 and 0.2 Mrad when combined with adequate refrigeration cause a considerable extension of storage life for many seafoods. However, a marked extension of their storage life might create a health hazard problem due to Clostridium botulinum type E. Three facts support this statement. First, ecological surveys in the United States and elsewhere have definitely established the ubiquity of C. botulinum type E in a marine environment. Second, radiation doses of the magnitude being considered have no major destructive effect on spores of this organism (7,8). Third, it possesses the ability to grow and to produce toxin as low as 38 F or 3.3 C (6).

Since haddock (Melanogrammus aeglefinus) and cod (Gadus callarius) are being considered by the AEC for possible commercial

irradiation, inoculated pack studies were undertaken to assess the health hazard risk of C. botulinum type E. This paper presents the inoculated pack data on haddock.

The concept of the inoculated pack to determine the margin of public health safety of radurized marine food products was discussed by Salvin, Ronsivalli, and Connors (10). Briefly, the rationale for such packs is an attempt to show that low dose irradiated seafoods pose no more health hazard than comparable unirradiated product. This involves the determination of the maximal expected product storage life and the earliest time of type E toxin production. The estimates of maximal product storage life are obtained by scientists at the Bureau of Commercial Fisheries Gloucester laboratory using an untrained consumer-type panel. The storage life values for unirradiated and irradiated product are designated as "X" and represent the time for unanimous rejection of a sample by this panel. Acceptance or rejection of a sample is based mainly on odor. The occurrence of unquestionable spoilage as recognized by the consumer appreciably ahead of the earliest time of possible type E toxin production would suggest a considerable margin of public health safety for radurized marine food products.

MATERIALS AND METHODS

Preparation and standardization of type E spore suspensions.

Spores of the Beluga type E strain were produced in TPG medium with incubation at 85 F (30 C) as described by Schmidt et al. (7). A low temperature spore suspension was produced in TPG medium with 1% added Yeast Extract (Difco) and incubation at 50 F (10 C). The suspensions were standardized by a replicated deep-tube technique using 5% Peptone (Difco) Agar medium and incubation at 85 F. Sodium thioglycollate at a concentration of 0.1% was employed for sporulation and in the standardization medium.

Inoculated packs. Freshly caught haddock were filleted, packed in ice and shipped by Air Freight to Chicago by Gloucester BCF personnel. The fillets were about 24 hr old when each experiment was started. Wide-mouth, 2 oz screw-cap jars were subjected to 250 F (121 C) for 5 min to destroy the non-heat resistant microflora. Circular plugs of fish were cut-out with a stainless steel die attached to a 1/4 inch drill press. The die was sanitized before use and the fillets were handled with rubber gloves to avoid any additional contamination. Each sample consisted of two plugs of fish having a total weight of about 40 g. Triplicate samples

were inoculated for each experimental variable. The inoculum (0.5 ml volume) was injected with a sterile syringe and needle at multiple sites to give 10^6 , 10^4 , or 10^2 spores/g. The 10^6 inoculum was preheated at 140 F (60 C) for 15 min to reduce possible toxin carryover. Unless stated otherwise, the 10^4 and 10^2 levels were not preheated.

Irradiation source. Samples were given 0.1 and 0.2 Mrad doses with a cobalt-60 source located at the Illinois Institute of Technology Research Institute. The source contained about 5,200 curries of cobalt-60 and delivered a dose rate of 6.24×10^4 rads/hr. The samples were irradiated at 40 F (4.4 C) to 42 F (5.6 C) in a temperature controlled chamber. Midway through the irradiation, the sample were rotated 180 degrees to equalize the dose. The samples were transported at wet ice temperatures prior to and after irradiation. The irradiated and inoculated unirradiated control samples were incubated at 50 F (10 C), 46 F (7.8 C), 42 F (5.6 C), or 40 F (4.4 C).

Sampling and toxin assays. At each designated sampling period, triplicate samples per variable were examined for odor, pH, and the presence of type E botulinum toxin. Each sample was blended with 80 ml of prechilled, sterile distilled

water to give a 1:3 blend. A portion of the blend was pipetted with a sterile cotton-plugged large bore pipette into a 20 x 150 mm screw-cap tube and frozen. The pH of the sample was determined on a second portion of the blend; in some cases, a total aerobic plate count was made with TPY agar (5). Finally, the sample was assayed for type E toxin using a sodium acetate buffer.

The acetate toxin assay procedure involved pipetting 2 ml of a blend into 2 ml of 0.5 M acetate buffer (pH 5.0) in a sterile 16 x 125 mm screw-cap tube. The buffered samples were mixed on a Vortex Mixer and held about 16 to 20 hr at 38 F (3.3 C). The buffer gave a final pH in the range 5.2 to 5.5. For digestion, 0.2 ml of freshly prepared trypsin (10%, Difco 1:250) was added. The sample was incubated at 98 F (37 C) for 3 hr. Duplicate mice were each injected intraperitoneally with 0.5 ml of the digest. In many instances, a simultaneous protection test was run; in others, the sample was assayed for toxin without antitoxin protection and toxin verified in a repeat test. The antitoxin used was obtained from the Pasteur Institute.

Blends assayed for type E toxin without trypsin digestion were diluted 1:2 with 0.5 M acetate buffer and held from 16 to 20 hr at 38 F (3.3 C) before being injected into mice. The lowest level of toxin theoretically detectable without or with trypsin digestion was 12 MLD/g. Nonspecific mouse deaths occurred with both unirradiated and 0.1 Mrad irradiated samples. To avoid nonspecific reactions, the antibiotics chloramphenicol and oxytetracycline were employed (9). Each mouse was given two intraperitoneal injections of the antibiotics, one approximately 20 hr before injection of the fish sample and the other when the sample was administered. Each antibiotic injection consisted of 0.1 ml volume containing 5.0 mg of Chloromycetin and 0.625 mg of Terramycin. The antibiotics were not used with 0.2 Mrad irradiated samples, since these samples failed to produce nonspecific toxicities.

RESULTS

Before the inoculated pack on haddock was started, a study was made to determine the sensitivity of the acetate toxin assay procedure. Frozen haddock and cod fillets were thawed, finely ground, and packed in 20 x 150 mm screw-cap tubes. These were inoculated with spores of the Beluga strain, subjected to 0.1 and 0.2 Mrad doses, vaspax sealed, and incubated at 40 F (4.4 C). Periodically during incubation, the samples were assayed for type E toxin. Assays were made without trypsin digestion and with trypsin digestion by the phosphate buffer and acetate buffer procedures. The comparative toxin assay results are shown in Table 1. Since the samples were incubated anaerobically, a gram-positive bacterial flora predominated that caused a gradual reduction from pH 6.6 to about 6.0. Sodium acetate buffer at pH 5.5 was used for the undigested samples and for the acetate samples to be trypsin digested. The results showed no advantage in testing for toxin by the phosphate buffer, trypsin digestion procedure, but rather strongly suggested the desirability of toxin assaying using the acetate buffer technique. The acetate procedure was adopted in the inoculated pack studies.

Table 2 shows the inoculated pack results on fresh haddock with inoculum levels of 10^4 and 10^2 spores/g and incubation at 50 F (10 C). The table is arranged to show the irradiation dose, the length of incubation, the fraction or multiple of X, and the number of toxic samples recovered. In all cases, samples reported as being nontoxic failed to show type E toxin without and with trypsin digestion. The inoculated unirradiated controls showed no detectable type E toxin at either inoculum level up to 2X. Samples inoculated with 10^4 spores and given 0.1 Mrad irradiation showed no toxin through 2X; however, similar irradiated samples inoculated with 10^2 spores showed toxin at 2X in one out of three replicates, but no toxin at earlier incubation times. Samples given 0.2 Mrad were definitely toxic at X and 2X with both inoculum levels, although comparable samples were nontoxic at 1/2X.

Not shown in Table 2 or in the tables to follow are the total aerobic counts, pH data, and our spoilage rejection determinations. Samples incubated at temperature intervals in the range 50 F (10 C) to 40 F (4.4 C) showed rapidly increasing total counts often reaching 10^8 bacteria or higher at storage times equal to 1/2X in many instances. Every sample showed a rapidly increasing pH, reaching pH 7.6 and, in some cases, as high as pH 8.4. Spoilage was characterized by the development of strong putrid odors in both unirradiated

and irradiated samples. The unirradiated samples invariably showed slime during spoilage, while the irradiated samples frequently showed mold growth in addition to slime growth. In general, our spoilage rejection times tended to coincide with the values reported by the BCF laboratory consumer panel.

The 46 F (7.8 C) assay results with 10^6 and 10^4 inoculum levels are shown in Table 3. The control and 0.1 Mrad samples showed no toxin at incubation times corresponding to 1/2X, X, and 2X. Samples inoculated with 10^4 spores and given 0.2 Mrad irradiation also were nontoxic at comparable values of X. But with a 10^6 inoculum level, 0.2 Mrad irradiated samples showed the presence of toxin at X and 2X in one out of three supposedly replicate samples.

The inoculated pack results shown in Tables 2 and 3 are based on spores produced at 85 F (30 C). Work in ground fish substrates at 40 F (4.4 C) and 42 F (5.6 C) suggested that spores of the Beluga strain produced at 50 F (10 C) possessed somewhat faster outgrowth ability at low incubation temperatures than spores produced at 85 F. An experiment

was conducted in fresh haddock with incubation at 42 and 40 F to compare the outgrowth ability of spores produced at 50 F to that of spores formed at 85 F. The comparative results are shown in Table 4. None of the unirradiated or the irradiated samples produced toxin up to 2X. Toxin assays conducted at 3/4X, X, and 1 1/2X also were negative. From these results, there appears to be no reason to expect faster spore outgrowth or growth at any lower temperature from spores produced at suboptimal temperatures.

To confirm the inoculated pack data on haddock incubated at 50 F (10 C) and 46 F (7.8 C), a second experiment was run. A new spore suspension of the Beluga strain was produced at 85 F (30 C) and used. All of the inoculum levels tested were preheated at 140 F (60 C) for 15 min. Unirradiated and irradiated samples were toxin assayed at 1/2X, 3/4X, X, 1 1/2X and 2X. The toxicity results are shown in Tables 5 and 6. As compared to the first experiment, unirradiated controls inoculated with 10^4 spores and incubated at 50 F showed toxin at 1 1/2X and 2X. In general, the results of the two experiments are comparable, although toxin was detected somewhat earlier with 0.2 Mrad irradiation in the second experiment. Table 7 summarizes the toxicity data of the second haddock experiment in terms of the earliest time for type E toxin production at 50 and 46 F.

DISCUSSION

Detection of low levels of type E toxin is complicated by the fact that the toxin is bound intracellularly and released primarily by vegetative cell lysis. It seems that type E cells do not lyse as readily when grown at low temperatures as they do at temperatures near the optimum for growth. Thus, a significant amount of toxin may pass undetected unless some means is used to extract the toxin from the cells or cause them to lyse.

The role of sodium acetate in the detection of low levels of type E toxin in fish samples is not clear. It may cause an extraction of toxin from intact cells, cause a release of possible adsorbed toxin on fish tissue, or simply establish a more favorable pH for toxin activation. In any event, it provides a convenient means of adjusting the sample pH for trypsin digestion.

In the conventional phosphate buffer trypsin digestion assay procedure, as described by Duff, Wright and Yarinsky (3), digestion is conducted with 0.067 M phosphate buffer at pH 6.0 to 6.2. This buffer is suitable for use with assays of broth cultures; however, it is a poor buffer for assays of food products showing high

pH values. Haddock fillets incubated aerobically or under partially aerobic conditions show a marked increase in pH. Unless the precaution is taken to adjust the pH for trypsin digestion, a significant amount of type E toxin may be destroyed. Use of the acetate buffer eliminates the necessity of making manual pH adjustments.

There appears to be little advantage in toxin assaying simultaneously with and without trypsin digestion. In the second experiment on haddock at 50 F (10 C) and 46 F (7.8 C), 180 samples were assayed both ways. With trypsin digestion, 32 samples showed detectable toxin versus 13 without digestion. In only two instances, toxin was detected without digestion from samples that failed to show toxin with digestion. These results suggest the desirability of first assaying with digestion and then retesting all apparently nontoxic samples without digestion.

The inoculated pack data on haddock with 0.1 and 0.2 Mrad irradiation at 42 F (5.6 C) and 40 F (4.4 C) suggest no type E hazard provided the product is distributed and stored at 42 F or below. This conclusion is supported by inoculum levels of 10^6 and 10^4 spores/g and spores produced at optimal and suboptimal temperatures.

At 50 F (10 C) and 46 F (7.8 C) incubation, the inoculated pack results with 0.1 Mrad irradiation appear favorable, since no toxin was detected up to twice the expected storage life with a 10^4 inoculum level. At 46 F, toxin was detected at X with a 10^6 inoculum level, but not at earlier sampling periods. We believe that negative toxin assay results with 10^4 spores/g for twice the maximal expected storage life would be ample evidence of the absence of a type E hazard, even though occasional toxin production might be detected earlier with a challenge dose of 10^6 spores per gram.

The results with 0.2 Mrad irradiation and incubation at 46 and 50 F are less favorable than those without irradiation or with 0.1 Mrad irradiation. Samples inoculated with 10^6 and 10^4 spores and given 0.2 Mrad irradiation showed type E toxin production at 1/2X with 46 F incubation. With 50 F incubation, samples inoculated with 10^4 and 10^2 spores were toxic at 1/2X and 3/4X, respectively.

Cann et al. (2) reported that vacuum packed herring, cod, and haddock given 0.3 Mrad irradiation and stored at 50 F (10 C) showed little differences in the time of type E toxin production from similar unirradiated fish. However, Ajmal (1) found that aerobically packed herring and cod subjected to 0.65 Mrad irradiation and incubated at 50 F were appreciably more susceptible to type E toxin

development than comparable unirradiated product. Our toxicity results on unirradiated and irradiated haddock in most cases are not based on equivalent incubation times. However, 0.1 and 0.2 Mrad irradiated haddock samples appeared to show toxin production somewhat earlier at 50 F and 46 F than corresponding unirradiated samples. When compared on the basis of equivalent X-values, 0.2 Mrad irradiated samples definitely showed toxin formation earlier than either 0.1 Mrad irradiated samples or unirradiated controls.

The reason for this apparent increased susceptibility of radurized fish to type E toxin production is unknown. Perhaps it is attributable to the reduction in the competitive microflora as Ajmal (1) suggests. However, it might be due to some favorable changes produced in the substrate by irradiation that stimulate faster outgrowth of type E spores. Another possibility is that low dose irradiation causes spore activation comparable to the heat activation effect observed with certain bacterial spores.

There were two instances in the inoculated pack experiments at 50 F (10 C) where type E toxin was detected with a 10^2 inoculum level in one out of three replicate samples, but none was detected

at a comparable time period with a 10^4 inoculum. Both cases occurred with 0.1 Mrad irradiation. Such results suggest that if additional replicates had been run with a 10^4 inoculum some samples might have shown toxin. The results illustrate the necessity of extensive replication to secure meaningful toxicity data.

The inoculated pack protocol followed for haddock will be extended to assess the public health safety of other marine food products of interest in the AEC program. Inoculated packs appear to be the best approach for determining the possible risk of growth and toxin production of C. botulinum type E in radurized refrigerated seafoods.

ACKNOWLEDGMENTS

This investigation was conducted under contract AT(11-1) 1183 with the Division of Biology and Medicine of the U. S. Atomic Energy Commission. The cooperative efforts of Louis J. Ronsivalli and Vincent Ampola of the Bureau of Commercial Fisheries Laboratory at Gloucester in supplying the fish fillets are gratefully acknowledged. The excellent technical assistance of Jerald K. Boltz of our laboratory is appreciated.

LITERATURE CITED

1. Ajmal, M. 1968. Clostridium botulinum type E: Growth and toxin production in food. J. Appl. Bact. 31:124-132.
2. Cann, D. C., B. B. Wilson, J. M. Shewan, T. A. Roberts, and D. N. Rhodes. 1966. A comparison of toxin production by Clostridium botulinum type E in irradiated and unirradiated vacuum packed fish. J. Appl. Bact. 29:540-548.
3. Duff, J. T., G. G. Wright, and A. Yarinsky. 1956. Activation of Clostridium botulinum type E toxin by trypsin. J. Bacteriol. 72:455-460.
4. Goresline, H. E., M. Ingram, P. Macuch, G. Macquot, D.A.A. Mossel, C. F. Niven, and F. S. Thatcher. 1964. Tentative classification of food irradiation processes with microbiological objectives. Nature, 204:237-238.
5. Pelroy, G. A., and M. W. Eklund. 1966. Changes in the microflora of vacuum-packaged, irradiated petrale sole (Eopsetta jordani) fillets stored at 0.5 C. Appl. Microbiol. 14:921-927.
6. Schmidt, C. F., R. V. Lechowich, and J. F. Folinazzo. 1961. Growth and toxin production by type E Clostridium botulinum below 40°F. J. Food Sci. 26:626-630.
7. Schmidt, C. F., W. K. Nank, and R. V. Lechowich. 1962. Radiation sterilization of food. II. Some aspects of the growth, sporulation, and radiation resistance of spores of Clostridium botulinum type E. J. Food Sci. 27:77-84.
8. Segner, W. P., and C. F. Schmidt. 1966. Radiation resistance of spores of Clostridium botulinum type E. Proc. Intern. Symposium on food irradiation. Karlsruhe, Federal Republic of Germany. Intern. Atomic Energy Agency, Vienna, 287-298.

9. Segner, W. P., and C. F. Schmidt. 1968. Nonspecific toxicities in the mouse assay test for botulinum toxin. Appl. Microbiol. 16: In press
10. Slavin, J. W., L. J. Ronsivalli, and T. J. Conners. 1966. Status of research and developmental studies on radiation pasteurization of fish and shellfish in the United States. Proc. Intern. Symposium on food irradiation. Karlsruhe, Federal Republic of Germany. Intern. Atomic Energy Agency, Vienna, 509-533.

TABLE 1. Comparative toxin assay results on irradiated fish samples incubated at 40 F (4.4C).¹

Substrate	Mrad	Fraction of samples toxin		
		U ²	AC ³	PB ⁴
Haddock	0.1	7/7	7/7	6/7
	0.2	8/9	9/9	4/9
Cod	0.1	7/12	9/12	0/12
	0.2	2/12	5/12	0/12
Totals		24/40	30/40	10/40

¹ Ground unsteamed product inoculated with 10⁶ spores/10 to 12 g sample.

² Assayed undigested by the acetate buffer procedure.

³ Trypsin assayed by the acetate buffer procedure.

⁴ Trypsin assayed by the conventional phosphate buffer procedure.

TABLE 2. Toxin assay results on haddock incubated at 50 F (10 C).

Mrad	Inc time (days)	X-value ¹	Fraction of samples toxic ²	
			10 ⁴ Inoc	10 ² Inoc
0	4	1/2	0/3	0/3
	8	1	0/3	0/3
	16	2	0/3	0/3
0.1	6	1/2	0/3	0/3
	12	1	0/3	0/3
	24	2	0/3	1/3
0.2	10	1/2	0/3	0/3
	20	1	3/3	2/3
	40	2	2/3	2/3

¹ BCF estimates of maximal product storage life under aerobic conditions.

² Number of samples containing type E toxin/number of samples assayed.

TABLE 3. Toxin assay results on haddock incubated at 46 F (7.8 C).

Mrad	Inc time (days)	X-value	Fraction of samples toxic ¹	
			10 ⁶ Inoc	10 ⁴ Inoc
0	6	1/2	0/3	0/3
	12	1	0/3	0/3
	24	2	0/3	0/3
0.1	6	1/2	0/3	0/3
	13	1	0/3	0/3
	26	2	0/3	0/3
0.2	12	1/2	0/3	0/3
	24	1	1/3	0/3
	50	2	1/3	0/3

¹ Number of samples containing type E toxin/number of samples assayed.

TABLE 4. Toxin assay results on haddock incubated at 42 F (5.6 C) and 40 F (4.4 C).

Temp °F	Expt ¹	Mrad	Inc time (days) ²	Fraction of samples toxic ³	
				10 ⁶ Inoc	10 ⁴ Inoc
42	1	0	29	0/3	0/3
		0.1	60	0/3	0/3
		0.2	82	0/3	0/3
	2	0	30	0/3	0/3
		0.1	60	0/3	0/3
		0.2	72	0/3	0/3
40	1	0	34	0/3	0/3
		0.1	82	0/3	0/3
		0.2	82	0/3	0/3
	2	0	34	0/3	0/3
		0.1	80	0/3	0/3
		0.2	80	0/3	0/3

¹ Experiment 1 conducted with Beluga spores produced at 85 F and experiment 2 with spores produced at 50 F.

² Incubation times represent 2X values.

³ Number of samples containing type E toxin/number of samples assayed.

TABLE 5. Toxin assay results for 50 F (10 C) incubated haddock
(experiment 2).

Mrad	Inc time (days)	X-value	Fraction of samples toxic			
			10 ⁴ Inoc		10 ² Inoc	
			U ¹	TD ²	U	TD
0	4	1/2	0/3	0/3	0/3	0/3
	6	3/4	0/3	0/3	0/3	0/3
	8	1	0/3	0/3	0/3	0/3
	12	1 1/2	0/3	1/3	0/3	0/3
	16	2	2/3	2/3	0/3	0/3
0.1	6	1/2	0/3	0/3	0/3	0/3
	9	3/4	0/3	0/3	0/3	0/3
	12	1	0/3	0/3	0/3	0/3
	18	1 1/2	0/3	0/3	0/3	1/3
	24	2	0/3	0/3	0/3	0/3
0.2	10	1/2	0/3	2/3	0/3	0/3
	15	3/4	0/3	3/3	0/3	1/3
	20	1	0/3	0/3	2/3	2/3
	30	1 1/2	2/3	2/3	1/3	0/3
	40	2	2/3	2/3	1/3	1/3

¹ Undigested.

² Trypsin digested.

TABLE 6. Toxin assay results for 46 F (7.8 C) incubated haddock (experiment 2).

Mrad	Inc time (days)	X-value	Fraction of samples toxic			
			10 ⁶ Inoc		10 ⁴ Inoc	
			U ¹	TD ²	U	TD
0	6	1/2	0/3	0/3	0/3	0/3
	9	3/4	0/3	0/3	0/3	0/3
	12	1	0/3	0/3	0/3	0/3
	18	1 1/2	0/3	0/3	0/3	0/3
	24	2	0/3	0/3	0/3	0/3
0.1	6	1/2	0/3	0/3	0/3	0/3
	9	3/4	0/3	0/3	0/3	0/3
	13	1	0/3	3/3	0/3	0/3
	18	1 1/2	0/3	0/3	0/3	0/3
	24	2	2/3	2/3	0/3	0/3
0.2	12	1/2	0/3	3/3	0/3	1/3
	18	3/4	0/3	2/3	0/3	1/3
	24	1	0/3	1/3	0/3	0/3
	36	1 1/2	0/3	1/3	0/3	0/3
	48	2	1/3	1/3	0/3	0/3

¹ Undigested.

² Trypsin digested.

TABLE 7. Summary of toxin assay results on haddock with incubation at 50 F (10 C) and 46 F (7.8 C). Experiment 2.

Temp °F	Inoc	Mrad	Inc time (days)	
			No toxin	Toxin
50	10 ⁴	0	8 (1)*	12 (1 1/2)
		0.1	24 (2)	---
		0.2	---	10 (1/2)
	10 ²	0	16 (2)	---
		0.1	12 (1)	18 (1 1/2)
		0.2	10 (1/2)	15 (3/4)
	10 ⁶	0	24 (2)	---
		0.1	9 (3/4)	13 (1)
		0.2	---	12 (1/2)
46	10 ⁴	0	24 (2)	---
		0.1	24 (2)	---
		0.2	---	12 (1/2)
	10 ⁶	0	24 (2)	---
		0.1	24 (2)	---

*-Values in parenthesis show fraction or multiple of X.