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Selecting methods for determining the presence of BoNT genes in New Zealand marine sediments

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Contents

1 Executive summary

Clostridium botulinum and other *Clostridium* species produce potent neurotoxins (BoNT) that cause botulism. These anaerobic organisms reside naturally in soils and marine sediments and can contaminate seafood. Given suitable storage conditions, they can grow and produce the toxin in the seafood, making consumers ill and even causing death.

Five BoNTs are known to affect humans – Types A, B, E, F and G – although there is little knowledge about Type G. They can all be produced by mesophilic organisms (unable to grow below 10°C) and some (Types B, E and F) can also be produced by psychrotrophic organisms (able to grow at refrigeration temperatures), which are of particular concern as they can grow under normal refrigerated storage conditions.

PCR (polymerase chain reaction) methods for detecting low numbers of organisms containing BoNT genes (Types A, B, E and F) from marine sediments were developed and evaluated with the following results.

- DNA was best extracted from spores using the FastPrep® bead-beating with lysozyme.
- The multiplex primers of Lindström et al. (2001) were more effective than the degenerate ones of Fach et al. (2002). The latter were found to lack specificity and gave false positive results under some conditions.
- When running the multiplex primers as a multiplex system, sensitivity was lost so we recommend using the primers singly and carrying out a separate PCR for genes producing each toxin type.
- The PCR was unable to detect low numbers of BoNT-producing organisms from marine sediments; it was easier to detect low numbers of vegetative cells than spores. To detect low numbers of spores it is necessary to first provide conditions in which they will germinate and multiply using an enrichment media.
- Using the Lindström et al. (2001) primer BoNT type A gave false positive results in approximately 50% of sediment samples. A nested PCR procedure was devised and this only gave 5% false positive results. Sequencing of the PCR product from the nested PCR is required to confirm positive results from the nested PCR.
- TPGY (Trypticase-Peptone-Glucose-Yeast extract broth) supported the growth of BoNT-producing *Clostridium* spp. better than did PYGS (Peptone Yeast Extract Glucose Starch broth).

- Adding lysozyme or a mixture of alanine and lactate did not improve spore germination.
- Temperatures between 25 and 35°C enriched both mesophilic and psychrotrophic BoNT-producing *Clostridium* spp. 30°C gave the best overall growth.
- Incubation for 2 weeks at 30°C is adequate to enrich low numbers of spores present in marine sediments, except for organisms that produce Type A toxin, where there were indications that recovery was improved after 5 weeks' storage.
- Pretreatment with 50% ethanol made it difficult to detect the presence of low numbers of spores among competitive flora while heating (10 min, 60°C) sometimes improved and sometimes reduced the ability to detect toxin-producing spores.
- Including trypsin in the enrichment media did not improve recovery of spores from the tested sediment sample.
- Freezing, thawing and frozen storage had minimal effect on the recovery of spores.

In summary, we propose that to test sediment samples for the presence of BoNT-producing organisms we will:

- carry out two replicate enrichments (TPGY at 30°C) on 10 g sediment samples, subjecting one to a heating pre-treatment (10 min, 60°C);
- collect 100 µL sub-samples (four in total) from the two enrichments after 2 and 5 weeks' storage;
- combine sub-samples, wash, resuspend and extract DNA using FastPrep® bead-beating with lysozyme;
- carry out separate PCR analyses for each of the four main BoNT Types (A, B, E and F) using the primers of Lindström et al. (2001);
- apply nested PCR for BoNT type A where bands are found matching the molecular weights of the control;
- if positive results are found, confirm their identity by sequencing the PCR products.

1.

2 Introduction

Clostridium botulinum is an anaerobic bacterium that produces an extremely potent neurotoxin (BoNT). BoNT is also produced by some other species of *Clostridium. Clostridium botulinum* has caused serious illness and death overseas when contaminated seafood products with an extended shelf-life, such as packaged smoked fish, has been consumed. Internationally the safety of smoked seafoods from botulism is assured by such things as creating high water phase salt, adding nitrites, using very high cook temperatures and/or avoiding vacuum packaging and modified atmosphere packaging with anoxic gas mixes. In contrast, New Zealand processors have generally assumed that the organism is sufficiently rare not to be a significant hazard and New Zealand seafood safety programmes have generally not been designed to control this organism. This has been a successful strategy in as much as there has never been a case of botulism from commercially produced New Zealand seafood. Against this, international codes of practice and regulations are increasingly being based on the assumption that the organism does present a significant hazard. If applied to New Zealand products, these codes of practice and regulations will put pressure on New Zealand seafood producers to introduce such measures as increasing the salt levels in their products, which would undesirable.

One of the first steps in a risk assessment is to evaluate the incidence of BoNT-producing organisms. Five BoNTs are known to affect humans – Types A, B, E, F and G – although Type G is little known. They can all be produced by proteolytic mesophilic organisms and some (Types B, E and F) can also be produced by non-proteolytic psychrotrophic organisms. The latter is of particular concern as these can grow under normal refrigerated storage conditions. Type E from psychrotrophic *C. botulinum* is recognised as a natural marine organism and is most commonly associated with seafood. Type B from psychrotrophic *C. botulinum* has also been found to prevail in some marine environments (Fach et al. 2002) but seafood has rarely been implicated in Type B botulism. Little is known about the frequency of the occurrence of BoNT-producing organisms in New Zealand, and this lack of information prevents an effective risk assessment. Organisms producing BoNTs of relevance to humans were not found in the few environmental samples that have been tested (Gill & Penney 1982) although one incident of Type A botulism has occurred (Flacks 1985). Given the lack of case history, BoNT-producing organisms are quite possibly absent from the New Zealand marine environment. Although Type E is endemic in marine sediments of Scandinavian waters (Huss 1981), it is unknown around the UK, probably because the prevailing currents are from the UK to Europe rather than vice versa (H. Huss, pers. comm.). Type E is also unknown in Australia despite several hundred samples having been tested (Szabo & Gibson 1997).

In 1998, the New Zealand Institute for Crop and Food Research Limited (C&FR) initiated a research programme funded by the Foundation for Research, Science and Technology to investigate the incidence of

BoNT-producing organisms in the New Zealand marine environment. We developed a sampling programme for marine sediments that we collected from areas favouring the ecology of the organism because international research had shown that the organism is more readily isolated from marine sediments than from seafood samples (Hielm et al. 1996). To simulate an underlying incidence of 5%, computer simulation was used to create 1 million sets of 500 samples, each sample having a 5% chance of being positive for botulism. In the 1 million sets of samples, the lowest number of positive results was 1 suggesting that if none of the 500 samples gave positive results there would be less than 1 in a million chance of a 5% incidence. The incidence of *C. botulinum* in areas where botulism occurs has been reported to range from 25 to 100% (Huss 1981). Thus, although it is impossible to demonstrate the total absence of the organism, its absence in the 500 samples would suggest that no particular emphasis need be placed on *C. botulinum* when developing food safety plans in New Zealand.

In 1998-99 we collected 498 sediment samples from the following harbours and coasts: Houhora Harbour, Rangaunu Bay, Rangaunu Harbour, Whangaroa Harbour, Hokianga Harbour, Bay of Islands, Whangarei Harbour, Kaipara Harbour, Kawau Bay, Mahurangi Harbour, Waitemata Harbour, Tamaki Strait, Firth of Thames, Coromandel Harbour, Manukau Harbour, Tauranga Harbour, Raglan Harbour, Kawhia Harbour, Marlborough Sounds and Otago Harbour. Samples were enriched in PYGS (Peptone Yeast Extract Glucose Starch broth) and incubated in anaerobic jars for 4 weeks at 20°C to allow *C. botulinum* to grow and produce toxin. Toxin was then assayed by mouse bioassay, the 'gold standard' for detecting *C. botulinum* toxin (Hudson 1996). None of the enriched samples produced toxin although similar enrichment techniques of such environmental samples from most other parts of the world have resulted in mouse deaths from human BoNTs (Hauschild & Dodds 1993).

No samples produced toxin but some positive control experiments (enrichments of sediment samples to which *C. botulium* spores had been added) also failed to produce toxin. At the time we had no way to confirm the identity of our positive control spore crops and derived the counts on the original titre as provided to us by MIRINZ. We have subsequently concluded that these spore crops had become contaminated and the failure to produce toxin was likely due to:

- the positive controls being diluted too much;
- the enrichment conditions used not being ideal for the organisms.

The original enrichments were disposed of but 20 g samples of the sediment samples were archived at -85°C to be used to confirm the mouse bioassay results.

The aims of our current research are to:

1. establish and validate an enrichment regime and PCR (polymerase chain reaction) genotyping technique for *C. botulinum* in the C&FR laboratory;
2. enrich the 500 archived sediment samples and test for the presence of BoNT genes and report the results to the New Zealand Food Safety Authority;

This report covers work towards the first of these objectives and includes the following steps to select a suitable PCR procedure:

1. evaluating 3 methods of extracting DNA from *Clostridium* spores;
2. evaluating 2 published sets of primers to uniquely identify BoNT genes from DNA and establishing suitable PCR conditions for identifying BoNT genes;
3. using the best conditions identified in steps 1 and 2, determine the detection limits for BoNT-producing spores and vegetative cells.

As this work showed that we could not detect BoNT genes from low numbers (e.g. <10 000) of spores, enrichment procedures designed to cause BoNT-producing spores to germinate and grow were evaluated. This consisted of determining:

2. 1. the effectiveness of 2 primary enrichment media (TPGY or PYGS);
3. 2. the impact of alanine and lactate or lysozyme on stimulating germination;
4. 3. the effect of temperatures between 15 and 35°C on germination and growth of BoNT-producing spores;
5. 4. the effect of ethanol and heat shock treatments to stimulate germination and eliminate competitive flora present in marine sediment;
6. 5. the effect of trypsin to reduce the effect of bacteriocin-producing competitors;
7. 6. because sediment samples have been stored frozen for a long time we are also evaluating the effect of freezing and thawing and frozen storage on spore germination and growth.

3 General methods

3.1 Anaerobic conditions

Media were deoxygenated by steaming and rapid chilling immediately before use. Inoculations, culture manipulations and dilutions were all performed as rapidly as practical in an aerobic environment with minimal homogenising or disturbance of the samples or media. Aerobic manipulations limited our ability to work with vegetative cells as these are relatively sensitive to oxygen while spores are reasonably resistant. In some cases (e.g. Table 6) manipulations of vegetative cells resulted in poor or no growth. This did not affect manipulations of spores (e.g. Table 1). Anaerobic incubation conditions were achieved in anaerobic jars (8 L) using three generators (BD GasPak EZ) and indicator strips. Indicator strips showed that anaerobisis in the headspace of the jars was achieved 2 and 4 h after the jar was closed. Subsequent experiments have shown that anaerobisis can consistently be achieved within 2 h by using 4 generators.

3.2 Enrichment media

Two main growth media used for *C. botulinum* were prepared in our laboratory.

3.2.1 TPYG (Trypticase-Peptone-Yeast-Glucose extract broth)

Amt	Ingredient	Source
5 g	Peptone	Difco
50 g	Tryptone	Difco
20 g	Yeast extract	Oxoid
4 g	Dextrose (D-Glucose)	BDH GP
1 g	Sodium thioglycollate	Sigma
1 L	Distilled water	

Dissolve all dry ingredients in water. pH = 7.0 ± 0.2. Autoclave 10 min @ 121°C. Refrigerate. Immediately before use, steam for 15 min and rapidly air chill. Reference: (Food and Drug Administration 1998).

3.2.2 *PYGS broth (Peptone Yeast Extract Glucose Starch)*

Amt	Ingredient	Source
5 g	Proteose peptone	Difco
5 g	Tryptone	Difco
10 g	Yeast extract	Difco
10 g	Meat extract	Oxoid > Lab Lemco'
5 g	Glucose	
1 g	Starch	Baker AR
0.5 g	Cysteine HCl	Sigma C7880
40 mL	Salts A	See below
40 mL	Salts B	See below
1 mL	Resazurin Soln	See below
1 L	Distilled water	

Dissolve all dry ingredients in water. Add Resazurin Soln, Salts A and Salts B solutions. Autoclave 15 min @ 121°C. Reference: Lund et al. (1990). To expunge oxygen this was steamed for 15 min and rapidly chilled immediately before use (Solomon & Lilly 1998).

3.2.3 *Salts Solution A*

Amt	Ingredient	Source	ChemStck #
0.265 g	$CaCl_2.2H_2O$		708
0.48 g	$MgSO_4.7H_2O$		151
2 g	NaCl		23
1 L	Distilled water	Micro Lab	

Add water to dry ingredients. Autoclave 15 min @ 121°C.

3.2.4 *Salts Solution B*

Amt	Ingredient	Source	ChemStck #
1 g	KH_2PO_4		45
1.3 g	$K_2HPO_4.3H_2O$		44
10 g	$NaHCO_3$		780
1 L	Distilled water	Micro Lab	

Add water to dry ingredients. Autoclave 15 min @ 121°C.

3.2.5 *Resazurin soln*

Amt	Ingredient	Source	ChemStck #
0.1 g	Resazurin		318
100 mL	Distilled water	Micro Lab	

3.3 *Clostridial strains*

Cultures of six BoNT-producing cultures on blood agar plates were supplied by MIRINZ, AgResearch.

Mesophilic organisms:

- A: *C. botulinum* Type A
- F: *C. botulinum* Type F
- But-E: *Clostridium butryricum* Type E

Psychrotrophic organisms:

- B17: *C. botulinum* Type B (17B)
- E-Beluga: *C. botulinum* Type E (Beluga)
- F202: *C. botulinum* Type F (202)

Additionally, a culture of *Clostridium sporogenes*, a Type A-like organism that does not produce toxin, was provided as a negative control.

3.4 *Control sediment sample*

To evaluate methods in the presence of natural marine sediment, sediment was collected from a single inter-tidal site in Blockhouse Bay, Auckland. The sediment was collected in the anaerobic zone, 2• 6 cm below the surface. All tests carried out on this sediment indicate that no BoNT genes were present. This test sediment sample was kept at 4°C until use.

3.5 *Enumeration methods*

3.5.1 *Plate count*

The method followed the general Guidelines for calculating and reporting APCs: (www.cfsan.fda.gov/~ebam/bam-3.html#conventional)

1. A serial dilution of each spore crop was carried out in iced cold saline (0.85% w/v NaCl) and then 2 x 0.1 mL of the each dilution were spread on to 2 plates of Columbia Blood Agar (Fort Richard).
2. The agar surface was dried for 10 min in a Class II Biohazard Cabinet, and then anaerobically incubated at 30°C for 72 h.
3. After incubation plates were counted (aiming to count colonies in dilutions with 25–250 colonies per spread plate).

4. Final count was calculated as a mean of the two replicate plates of dilutions giving 25–200 colonies and reported as colony forming units (cfu) per mL.

3.5.2 Haemocytometer

We followed the method described in "Using a Counting Chamber Method" (www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html):

Briefly:

- To prepare the counting chamber the mirror-like polished surface is carefully cleaned with lens paper. The coverslip is also cleaned.
- The coverslip is placed over the counting surface prior to putting on the cell suspension. The suspension is introduced into one of the V-shaped wells with a pipette. The area under the coverslip fills by capillary action. Enough liquid is introduced so that the mirrored surface is just covered.
- The charged counting chamber is then placed on the microscope stage and the counting grid is brought into focus at low then high power. Cell suspensions should be dilute enough so that the cells do not overlap each other on the grid, and should be uniformly distributed. Sometimes dilution is required.
- Cells are systematically counted in selected squares so that the total count is 100 cells or so (number of cells needed for a statistically significant count).
- To determine the final count in cells/mL, first divide the total count by 0.1 (chamber depth) then divide the result by the total surface area counted. If the cell suspension was diluted to get the cell density low enough for counting, multiply the final count by the dilution factor.

3.5.3 MPN

Although the plate counts and haemocytometer counts gave good reproducible results on replicate samples, they are not very amenable to selectively distinguishing BoNT-producing organisms from non-toxic ones. We also knew that increasing BoNT-producing organisms in sediment samples was going to require enrichment in broths so the less accurate most probable number (MPN) method, which uses serial dilutions of broths, was also used for enumeration.

The basic method is described in "Most Probable Number (MPN)" www.cfsan.fda.gov/~ebam/bam-a2.html

1. Serial dilutions (4 x 180 µL) were performed using enrichment broth (e.g. TPYG or PYGS) in 96 well plates that were incubated (e.g. 96 h, 30°C).
2. After incubation the wells were read by eye (turbidity) and the cloudy wells were recorded as positive and clear wells as negative, or by using an electronic plate reader (Multiskan Ex, Thermo Electronic Co-operation).

3. To enumerate BoNT-producing organisms among other contaminating organisms, the PCR method developed below was applied to a sub-sample from relevant wells of the plate.
4. MPNs from the PCR results from mixed cultures, or the turbidity results from pure cultures, were calculated from the MPN tables, BAM-MPN Calculator (www.cfsan.fda.gov/~ebam/bam-a2.html#excl) Haldane's Approximation to the standard errors of the $\log_{10}$ MPNs as generated by the BAM-MPN Calculator were used to compare results. Results for which standard errors did not overlap were considered to be significantly different from each other.

3.6 Spore crops

Spore crops were prepared using a two-phase sporulation medium (Peck et al. 1992).

3.6.1 Solid medium preparation

1. Add 300 mL of water into 500 mL bottle.
2. Add 30 g of meat extract (Oxoid; CM0081).
3. Add 4.5 g of agar and 0.3 g of glucose immediately before autoclaving.
4. Autoclave at 121°C for 15 min, then rapidly air chill and use.
5. If not used when chilled, immediately before use, steam for 15 min, rapidly air chill then use.

3.6.2 Preparation of the inoculum

1. Inoculate 10 mL of pre-reduced TPYG broth with the BoNT-producing culture and incubate at 30°C for 24 h.
2. Autoclave 40 mL of sterile water to deoxygenate it.
3. Add the deoxygenated water (40 mL) and the grown inoculum (10 mL TPYG) on to the pre-reduced solid medium.
4. Put the bottles into an anaerobic jar.
5. Loosen the bottle caps, add anaerobic generator sachets, close the jar and incubate at 30°C for 2–6 weeks using indicator strips to confirm anaerobic conditions were maintained.
6. Occasionally open jar, withdraw a sample and check microscopically to monitor bacterial growth and percentage sporulation. If less than 70% spores, continue anaerobic incubation.

3.6.3 Harvesting

1. Concentrate liquid phase of two phase medium by centrifugation at 4300 x g for 15 min.
2. Wash 4 times with iced cold saline (0.85% w/v NaCl).
3. Resuspend in 10 mL of iced cold saline (0.85% w/v NaCl).

4. Sonicate in ultrasonic water bath (ultrasonics Pty Ltd, Manly Vale, NSW, Australia) 10 min at 40 kHz 100 W output at 10°C to release spores from sporangia and wash with iced cold saline (0.85% w/v NaCl).
5. Repeat the above step 3 times.
6. Centrifuge at 4300 g for 15 min.
7. Resuspend the above (cell-free spores) in 5 mL iced-cold saline (0.85% w/v NaCl).
8. Store spore crops at 2°C.
9. Enumerate by performing serial dilution and plate count on Columbia Blood Agar, MPN using TPYG broth and Haemocytometer Counts.
10. Both the blood plate count and MPN were performed before and after heat- shocking at 60°C for 10 min.
11. Blood agar and MPN plates were anaerobically incubated at 30°C for 72 h.
12. After incubation the blood agar plates were counted and MPN wells were read by a Multiskan Ex (Thermo Electronic Co-operation Plate Reader).

3.6.4 Spore crop counts

Table 1: Botulinum spore crop counts.

Spore strain	Haemocytometer count (cells/mL)	Plate count No heat (CFU/mL)	Plate count Heated1 (CFU/mL)	MPN/mL No heat	MPN/mL Heat1	Cell count used for PCR sensitivity (cells/mL)
A	7.0×10^7	7.5×10^7	7.5×10^7	1.70×10^7	2.00×10^7	7.5×10^7
B17	1.5×10^8	1.2×10^8	4.6×10^7	2.00×10^7	1.30×10^6	1.2×10^8
F	3.5×10^9	9.2×10^9	6.3×10^9	1.30×10^{10}	1.30×10^{10}	9.2×10^9
F202	4.0×10^7	4.5×10^7	2.4×10^7	6.1×10^6	3.40×10^6	4.5×10^7
But-E[2]	7.5×10^8 8.3×10^8	- 2.7×10^7	- 1.2×10^7	1.05×10^9 1.21×10^7	9.04×10^6	7.5×10^8
E-Beluga[2]	6.6×10^8 1.7×10^9	- 2.6×10^7	- 2.0×10^7	3.75×10^9 3.90×10^7	9.04×10^6	6.6×10^8

[1]Spore crop heated (10 min, 60°C) and cooled before counting.
[2]Second row of counts counted after 8 months' anaerobic storage at 2°C.

4 *Extraction of DNA from* Clostridium *species*

4.1 *Method*

Four methods of DNA extraction were compared:

1. Bead-beating on a vortex
2. FastPrep® bead-beating
3. FastPrep® bead-beating with lysozyme treatment
4. Extraction using the commercial DNeasy tissue kit.

Enriched spore samples and control sediment culture seeded with pure culture (ca 10^7 cell/mL) were used for DNA extraction. The samples were mixed thoroughly by vortexing and 2 mL of each sample was taken and transferred to a 2-mL Screw-top Micro tube (Cat. #72693005, Sarstedt, Germany). The samples were centrifuged (8000 x g, 5 min) and the supernatant removed. The pellet was re-suspended in 0.1 M phosphate buffer (270 µL, pH 7.4) (with 30 µL of 50 mg/mL added lysozyme for method 3). The suspension was mixed by vortexing and incubated (37°C, 30 min) followed by the addition of Lysis buffer (300 µL, 0.1 M NaCl, 0.5 M Tris, pH 8.0, 10% SDS) and 300 µL chloroform: isoamyl alcohol (24:1). The suspension was mixed and transferred into a 2-mL Screw-top Micro tube (Cat. #72693005, Sarstedt, Germany) containing 100 mg of 0.1 mm Zirconia/silica beads (Catalog #110791012, Biospec products Inc.) and 100 mg of 2.5 mm Zirconia/silica beads (Catalog #110791252, Biospec products Inc.). In method 1, the mixture was vortexed (1 min, maximum speed) on a standard laboratory vortex instrument (MS1 Minishaker, IKA®, Guangzhou, China). For methods 2 and 3 the mixture was run on a FastPrep® FP220A Instrument (Qbiogene, CA, USA) at 4.5 m/s for 40 seconds. Method 4 followed the instructions of the commercial DNeasy tissue kit (Cat. #69506, Qiagen, Hilden, Germany). The suspension was centrifuged (13 000 x g, 8 min). The supernatant (about 700 µL) was transferred to an Eppendorf tube followed by addition of 7 M NH_4Ac (390 µL). The suspension was mixed by vortexing and centrifuged at 13 000 x g for 8 min. The supernatant (about 830 µL) was transferred to another Eppendorf tube, isopropanol (448 µL) added and thoroughly mixed by repeatedly inverting of the tube. The mixture was left to stand (room temperature, 1 min) and then centrifuged (3000 x g, 10 min). The supernatant was decanted and the pellet was washed with ice-cold ethanol (750 µL), centrifuged (13 000 x g, 10 min) and the DNA in the pellet air-dried and re-suspended (100 µL H_2O). This was stored (4 or -20°C) for future use.

4.2 Results

The electrophoresis of DNA samples extracted from the seeded sediment enrichments by FastPrep® bead-beating with lysozyme treatment (method 3) showed the highest recovery of genomic DNA, with bands of abundant DNA over 12 kb in size, compared to the other two methods (Figure 1). The DNA extracted by bead-beating on the laboratory vortex (method 1) showed severe shearing of the genomic DNA due to the shear force of the vortexing, resulting in DNA with smaller molecular weight and lower DNA quantity. The DNA samples extracted by use of the DNeasy tissue kit (method 4) had lowest genomic DNA recovery.

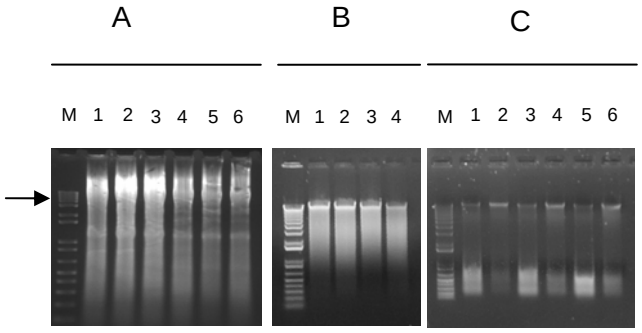

Figure 1: Yield of DNA extraction using different approaches for C. botulinum strains grown in PYGS broth containing 10% sediment (w/v). DNA samples (5 µL each lane) were analysed by electrophoresis on 1% agarose gel. M: 1 kb plus DNA ladder with maximum molecular weight of 12 kb (arrow) (Invitrogen).

A: DNA extracted by FastPrep® bead-beating. Lanes 1 and 4: Sediment culture of *C. butyricum* Type E; Lanes 2 and 5: Sediment culture of *C. botulinum* Type E Beluga; Lanes 3 and 6: Spent control sediment cultures seeded with ca 2 x 10^7 cells of *C. butyricum* Type E. Lanes 1, 2, and 3 were treated with lysozyme (method 3). Lanes 4, 5, and 6 were extracted without lysozyme treatment (method 2).

B: DNA extracted by bead-beating on vortex with lysozyme treatment. Lane 1: Sediment culture of *C. botulinum* Type A; Lane 2: Sediment culture of *C. botulinum* Type E beluga; Lane 3: Sediment culture of *C. botulinum* Type F; Lane 4: Spent control sediment culture seeded with ca 2 x 10^7 cells of *C. botulinum* Type A.

C: DNA extracted by commercial DNeasy tissue kit. Lanes 1 and 2: Sediment culture of *C. butyricum* Type E; Lanes 3 and 4: Sediment culture of *C. botulinum* Type E beluga; Lanes 5 and 6: Spent control sediment culture seeded with ca 2 x 10^7 cells of *C. butyricum* Type E. Lanes 1, 3, and 5 were first eluents in 50 µL H_2O. Lanes 2, 4, and 6 were second eluents in 50 µL H_2O.

4.3 DNA extraction conclusion

FastPrep® bead-beating with lysozyme treatment showed the greatest recovery of genomic DNA over the other DNA extraction approaches (FastPrep® bead-beating without lysozyme treatment, bead-beating on vortex with lysozyme treatment, and commercial DNeasy tissue kit). This method of DNA extraction is used in subsequent work and proposed for use with real samples.

5 Selection of primers and PCR conditions

5.1 Primers

Published primers from two sources (Lindström et al. 2001; Fach et al. 2002) were evaluated in this study. Primer pairs targeted to the BoNT genes of either *C. botulinum* Types A, B, E, or F were used (Tables 2 & 3). Fach et al.'s primers (Table 2) were designed as degenerate primers (CB1 and P261) that could simultaneously detect any of the 4 BoNTs in a single PCR band. Lindström et al.'s primers (Table 3) were designed as multiplex primers of different molecular weights so that different BoNTs could be detected as different PCR bands in a single PCR reaction.

Table 2: Location of primers within the specified genes (Fach et al. 2002).

		Location within gene (accession no. in GenBank):			
Primers	Sequences a	BoNT/A (X73423)	BoNT/B (M81186)	BoNT/E (X62683)	BoNT/F (M92906)
CB1	5'- AWAATAATTCRGGATGGAAARTATC -3'	3002–3026	2926–2950	3061–3085	3070–3094
P261b	5'- KRTATRYHHRATCDTYHTTTCTAAC -3'	3616–3640	3537–3561	3650–3675	3684–3708
CBA b	5'-CACTACGCTACCTCTAGGCCC-3'	3508–3528			
CBB b	5'-GAATTTTGATTATATTTGCTACGTG-3'		3424–3448		
CBE b	5'-GCTTCTTATATTATTAATGCTTAAAGTAG-3'			3538–3566	
CBF b	5'-CTATAGGACCATTTTTTCTTATAATGAC-3'				3633–3660

a In the sequence of oligonucleotides, W is (A, T), R is (A, G), K is (G, T), Y is (C, T), H is (A, T, C), D is (G, A, T), and M is (A, C).
b Oligonucleotide positioned in the complementary strand.

Table 3: Primers for PCR detection of C. botulinum *Types A, B, E, and F (Lindström et al. 2001).*

Type	Primer		Sequence (5'-3')	Product size (bp)	Coding region
	CBMLA1	Forward	AGC TAC GGA GGC AGC TAT GTT 782 1788–1808	782	1788–1808
A	CBMLA2	Reverse	CGT ATT TGG AAA GCT GAA AAG G		2569–2548
	CBMLB1	Forward	CAG GAG AAG TGG AGC GAA AA 205 434–453	205	434–453
B	CBMLB2	Reverse	CTT GCG CCT TTG TTT TCT TG		638–619
	CBMLE1	Forward	CCA AGA TTT TCA TCC GCC TA	389	156–175
E	CBMLE2	Reverse	GCT ATT GAT CCA AAA CGG TGA		544–525
	CBMLF1	Forward	CGG CTT CAT TAG AGA ACG GA	543	185–194
F	CBMLF2	Reverse	TAA CTC CCC TAG CCC CGT AT		727–708

A search of the genebank showed that the Lindström el al. primers are specific for their respective organisms within the bacterial kingdom. Due to the large number of variable nucleotides, the degenerate primers of Fach et al. could not be tested in the genebank. Evaluations were carried out on both sets of primers as described below.

5.2 PCR conditions

PCR amplifications for BoNT genes were carried out with the degenerate primers (CB1 and P261) and with the specific primers listed in Table 2 following the general procedures described by Fach et al. (2002). The PCR mix contained 2 µL of 10 x PCR buffer, 2.5 mM $MgCl_2$, 0.25 µM of each forward and reverse primers, 200 µM of each deoxynucleoside triphosphate, 0.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, USA), in a total volume of 20 µL. A series of 10-fold dilutions were made for DNA extracted by FastPrep® bead-beating (method 3). Samples (1 µL) from each of the dilutions were used as DNA templates for PCR amplification. Bovine serum albumin (BSA, 1 µL, 30 mg/mL) was added to evaluate the effect of inhibitor in the DNA samples. The following temperature profiles were applied: 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 7 min. PCR products (5 µL) were analysed by gel electrophoresis in 1% agarose gels.

PCR with the primers designed by Lindström et al. (2001) was performed in a total volume of 20 µL containing 2 µL of template, 0.3 µM concentrations of each primer, 2 µL of 10 x PCR buffer, 2.5 mM $MgCl_2$, 220 µM concentrations of each deoxynucleotide triphosphate, 0.5 U of DNA polymerase (Invitrogen, Carlsbad, USA). The PCR temperature profiles were: 1 cycle of 95°C for 3 min; 35 cycles of 95°C for 30 s, 60°C for 25 s, and 72°C for 60 s; and 1 cycle of 72°C for 3 min. The amplified PCR products (5 µL) were visualised in 1% agarose gels by electrophoresis.

To determine the detection limits of the PCRs for spores, spore crops were diluted in phosphate buffer solution (0.1 M, pH 8.0) by 10-fold serial

dilutions. One hundred µL of diluted spore solution from each dilution of each spore crop were used for DNA extraction by FastPrep® bead-beatingas described above. The extracted DNA pellets were eluted in 20 µL water. The DNA samples extracted from the spores of least dilute extract from each spore crop were also diluted in water by 10-fold serial dilutions. One µL of each diluted or undiluted DNA samples were used for PCR amplification. Ten µL of the resulting PCR products were loaded for electrophoresis analysis in 1% agarose gels (Tables 4 & 5).

To determine the detection limit for vegetative cells, 0.1 mL of each spore crop was inoculated into 10 mL TPYG and incubated at 30°C for 72 h to produce vegetative cells. These were quantified by blood agar plate counts, MPNs and by haemocytometry. Cells were then serially diluted in phosphate buffer (0.1 M, pH 8.0) and a 30 µL sample from each dilution taken for DNA extraction by FastPrep® bead-beating. Two µL from each DNA sample was used for PCR amplification. Ten µL of PCR product was loaded for electrophoresis analysis in 1% agarose gels.

5.3 Results

5.3.1 Amplification of BoNT genes using primers of Fach et al. (2002)

PCR amplification of BoNT genes are shown in Figures 2 & 3. The results show that PCR amplification of BoNT genes using the forward degenerate primer CB1 with the reverse gene-specific primers was more sensitive than that using both degenerate primers CB1 and P261 (Figure 2).

To examine the existence of inhibitors in the DNA samples from enriched sediment culture of the BoNT-producing strains, BSA was introduced in the PCR reaction at a final concentration of 1.5 mg/mL with the DNA from enriched sediment cultures of *C. botulinum* Type E beluga and *C. butyricum* Type E. Results showed that inhibitor(s) were present in the extracted DNA for dilutions of up to 1 in 10, as no PCR amplification was found in the undiluted or 10-fold diluted DNA samples when no BSA was added in the PCR reaction (Figure 3: Lanes 1, 9, and 10 in part A, and Lane 1 and 2 in part B), compared to the PCR reaction with addition of BSA (Figure 3: Lanes 9–15 in part B). However, the detection limit of the PCR with BSA was higher than that without BSA (one 10-fold dilution). This suggests that addition of BSA eliminates the inhibition in the DNA sample, but adversely reduces the detection limit of the PCR.

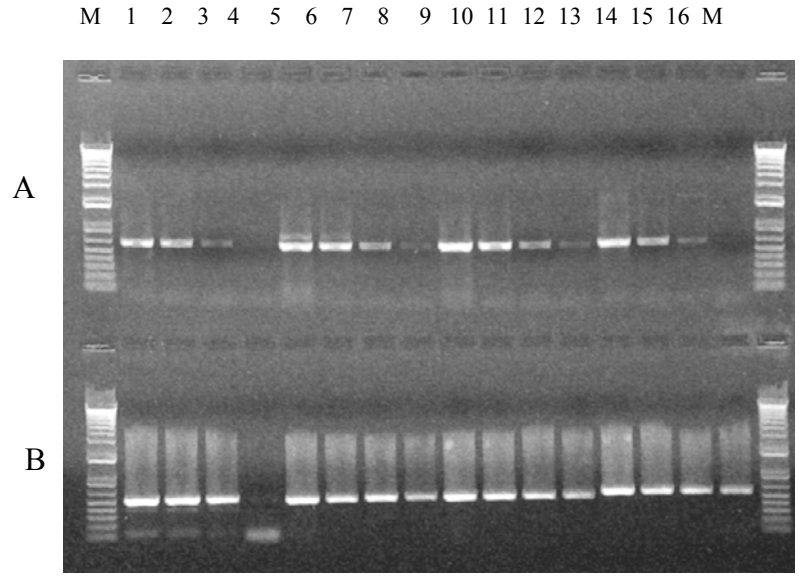

Figure 2: Comparison of sensitivity of PCR amplification of BoNT genes using degenerate primers CB1 & P261 (Part A), and CB1 and gene-specific reverse primers (Part B). Lanes A1• A4: undiluted DNA and 10-1, 10-2, 10-3 dilutions of DNA extracted from culture of C. botulinum *Type A; Lanes A5• A8: undiluted DNA and 10-1, 10-2, 10-3 dilutions of DNA from* C. butyricum *Type E; Lanes A9• A12: undiluted DNA and 10-1, 10-2, 10-3 dilutions of DNA from of* C. botulinum *Type E beluga; Lanes A13• A16: undiluted DNA and 10-1, 10-2, 10-3 dilutions of DNA from* C. botulinum *Type F; Lanes B1• B4: undiluted DNA and 10-1, 10-2, 10-3 dilutions of DNA extracted from culture of* C. botulinum *Type A; Lanes B5• B8: undiluted DNA and 10-1, 10-2, 10-3 dilutions of DNA from* C. butyricum *Type E; Lanes B9• B12: undiluted DNA and 10-1, 10-2, 10-3 dilutions of DNA from of* C. botulinum *Type E beluga; Lanes B13• B16: undiluted DNA and 10-1, 10-2, 10-3 dilutions of DNA from* C. botulinum *Type F. M: 1 kb plus DNA ladder (Invitrogen).*

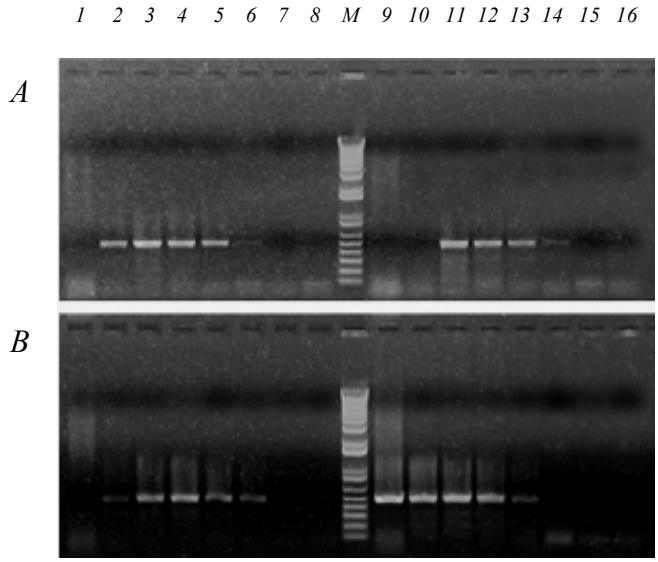

Figure 3: Evaluation of BSA in eliminating the inhibitor in the DNA samples from enriched sediment by PCR. PCR products (5 µL) were analysed by electrophoresis. Lanes A1• A8: undiluted DNA and 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7 dilutions of DNA extracted from sediment culture of C. butyricum *Type E; Lanes B1• B8: undiluted DNA and 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7 dilutions of DNA extracted from a sediment culture of* C. botulinum *Type E beluga; Lanes A9• A16 and B9• B16: undiluted DNA and 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7 dilutions of DNA extracted from a control sediment culture (without inoculation of* C. botulinum*) seeded with ca 2 x 107 cells of* C. butyricum *Type E before DNA extraction; Lanes A1• A16 and Lanes B1• B8: PCR without addition of BSA; Lanes B9-B16: PCR with addition of BSA (30 mg/m) at a final concentration of 1.5 mg/mL. M: 1 kb plus DNA ladder (Invitrogen).*

During the course of the research, non-specific amplification from the primers of Fach et al. (2002) was found on various occasions, as illustrated in Figure 4. Amplifications by primers (CB1 & CBA) for the Type A BoNT gene were obtained in DNA samples from colonies of *C. botulinum* Type F on blood agar sub-cultured from broth cultures at 30°C (Figure 4: Lanes 5–7) with the size similar to that of PCR product by CB1 and CBA obtained from *C. botulinum* Type A (Figure 4: Lane 4). This was observed whenever Type F was amplified with primer CB1 and CBA. DNA sequencing of PCR products by Type A BoNT-specific reverse primer CBA from two colonies of Type F found that the sequences were identical (Figure 5). However, a similarity search against the NCBI database using the Basic Local Alignment Search Tool (BLAST) programme (Altschul et al. 1997), available on the internet (/www.ncbi.nlm.nih.gov/BLAST/) found that these sequences do not match any existing sequences in the GenBank. This result demonstrates that these sequences are new and have not previously been reported. Furthermore, it shows that the primers CB1 and CBA are not uniquely specific for the Type A BoNT gene.

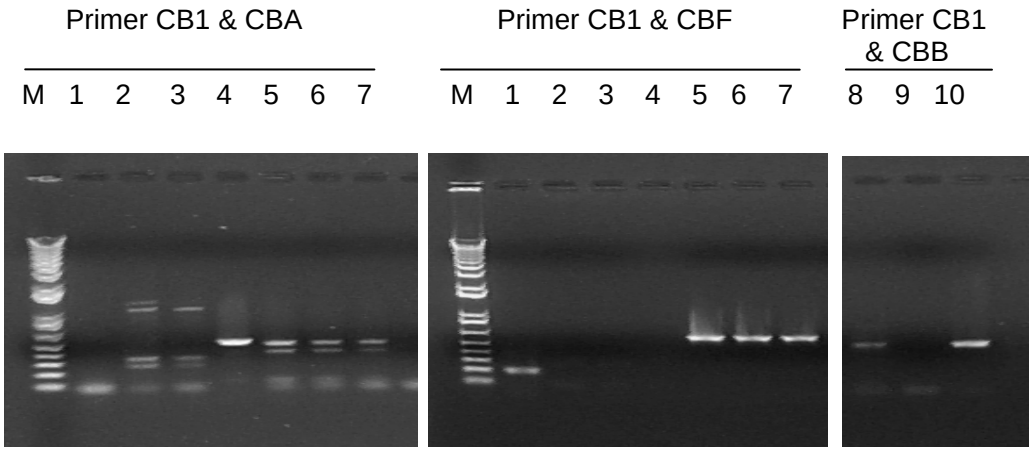

Figure 4: Specificity testing of primers CB1 & CBA, CBB, and CBF by PCR for DNA samples from colonies on blood agar sub-cultured from broth cultures at 30°C. Lanes 1–3: C. butyricum *Type E; Lane 4:* C. botulinum *Type A; Lanes 5–7:* C. botulinum *Type F. Lanes 8–9: colonies from a contaminated broth culture of* C. botulinum *Type E beluga; Lane 10:* C. botulinum *Type 17B. M: 1 kb plus DNA ladder (Invitrogen).*

```
C6A     --WWAWTWAT TCRGGATGGA AARTATCTGT GGCCTCAGAG GTCTTAAGTA
C7A     --WWAWTWAT TCRGGATGGA AARTATCTGT GGCCTCAGAG GTCTTAAGTA

C6A     CTCCTAAGTA TTCTATAGGG TTAAATCTTY TAAATGCTAA AGCTACYTTA
C7A     CTCCTAAGTA TTCTATAGGG TTAAATCTTY TAAATGCTAA AGCTACYTTA

C6A     GCTACAGAGG AACTATTTGC ATGGACTATA GTGGTGGTTT TACTTAGTCT
C7A     GCTACAGAGG AACTATTTGC ATGGACTATA GTGGTGGTTT TACTTAGTCT

C6A     TATATTTGAA AAAATATTTA GATATTATAT TAAAAAAGTA TTTTGAGCAA
C7A     TATATTTGAA AAAATATTTA GATATTATAT TAAAAAAGTA TTTTGAGCAA

C6A     AAGGTAGTGT ATCTTTATAA TATTAATGTT AGTGTATTTA GTAGTACATA
C7A     AAGGTAGTGT ATCTTTATAA TATTAATGTT AGTGTATTTA GTAGTACATA

C6A     TGAGAATTTT TAAGAAGAGA GTCTGAAATT AAGAATTACT TAATATAGAG
C7A     TGAGAATTTT TAAGAAGAGA GTCTGAAATT AAGAATTACT TAATATAGAG

C6A     ATAAAATTAT GAAAAAACAT GAGGTAGTGT ATGAAATGCA ATTTATAAAT
C7A     ATAAAATTAT GAAAAAACAT GAGGTAGTGT ATGAAATGCA ATTTATAAAT

C6A     GCTTGTAAAG CTTATGGAGA TAAAATAATT TTGAAGGATT TAAATTTAAA
C7A     GCTTGTAAAG CTTATGGAGA TAAAATAATT TTGAAGGATT TAAATTTAAA

C6A     CTTTGMAAAG AATAAAATAA CTGCTCTGTT AGCTCCCTTG CKKGGGTC
C7A     CTTTGAAAAG AATAAAATAA CTGCTCTGTT AGCTCCTTCG TGGGKG—
```

Figure 5: Sequences and alignment of PCR products by primers CB1 & CBA from DNA samples from colonies of C. botulinum *Type F on blood agar sub-cultured from broth cultures at 30°C. Underlined sequences correspond to the primer CB1.*

Non-specific amplification was also obtained using primers CB1 and CBB in the DNA sample (C8B) from a contaminated broth culture of *C. botulinum* Type E beluga with the size of the PCR product similar to that from *C. botulinum* Type 17B. The DNA sequence of this PCR product was obtained by sequencing using Type B BoNT-specific reverse primer CBB. A similarity search against the NCBI database found that the sequence was close to that of the *thdF* gene of *Bacillus cereus* E33L (Accession number: CP000001) (Figure 6). This shows that the primers CB1 and CBB are also not uniquely specific for the Type B *BoNT* gene.

```
C8B          -WW------- ---------- ---------- ---------- ---AWTWATT
B. cereus    TGGGAATAAG TTTAGTGTTT TTACCATGGG ATATAATAAA TTGAGTTATT

C8B          CR-------- ------GG-- -----ATGGA ---------- ----------
B. cereus    CTTCCTTTGG TAGATAGGTT CGTTAATGGA TCCCTAATAA TTTAGTGAAA

C8B          -AARTA-TCA TGTTGAAATA GAGATAAATA AGCAAGTGAG GTGAAAGGAC
B. cereus    GAAATAATCA TGTTGAAATA GAGATAAATA AGCAAGTGAG GTGAAAGGAC

C8B          ATGGAATTTG ATACAATTGC CGCGATTTYC ACAGCGCTTG GAGAGGGTGC
B. cereus    ATGGAATTTG ATACAATTGC TGCGATTTCC ACAGCGCTTG GAGAGGGTGC

C8B          AATTGCCATT GTTYGAGTAA GTGGGGATGA TGCGGTTGAA AAAGTTAATY
B. cereus    AATTGCCATT GTTCGAGTAA GTGGGGATGA TGCGGTTGAG AAAGTTAATC

C8B          GTATTTTTAA AGGGAAGGAT TTAACAGAGG TTCCTTYTCA TWCAATTCAT
B. cereus    GTATTTTTAA GGGGAAGGAT TTAACAGAGG TTCCTTCTCA CACTATTCAT

C8B          TATGGTCATA TTGTTGATTT AGATACAAAT CAGGTTATTG AAGAAGTAAT
B. cereus    TATGGTCATA TTGTTGATTT AGATACAAAT CAAGTTATTG AAGAAGTTAT

C8B          GGTGTCCATT ATGCGTGCAC CAAGGACTTT TACACGTGAA AATATAGTAG
B. cereus    GGTGTCTATT ATGCGCGCAC CAAGGACTTT TACACGTGAA AATATAGTGG

C8B          AAATTAACTG TCACGGTGGA CTTGTTTCGG TAAWCAAAGT ATTACAGCTT
B. cereus    AAATTAACTG TCACGGTGGA CTTGTTTCAG TAAATAAAGT ATTGCAACTT

C8B          ATTTTAGCGC AAGGGGTAAG ATTAGCGGAG CCTGGTGWAT TTACAAAACG
B. cereus    ATTCTAGCAC AAGGAGTACG ATTGGCAGAA CCGGGCGAAT TTACAAAACG

C8B          TGCTKTTTTA AATGGCCGTA ATGATTWWTC ACRARWAGAA GCWGYGMRAR
B. cereus    TGCTTTTTTA AATGGACGTA TCGATTTATC ACAAGCAGAA GCTGTTATGG

C8B          WMTWWA---- ---------- -ATT
B. cereus    ATTTGATTCG AGCAAAAACA GATC
```

Figure 6: Sequence of PCR product (C8B) by primers CB1 & CBB from DNA sample from colony of C. botulinum *Type E beluga on blood agar sub-cultured from broth cultures at 30°C, and the alignment with the thdF gene of* B. cereus *E33L (Accession number: CP000001). Underlined sequences correspond to the primer CB1.*

Due to their lack of specificity, we do not recommend using the primers of Fach et al. (2002) and only recommend using those of Lindström et al. (2001). Only the latter were used for subsequent work.

5.3.2 *Amplification of BoNT genes using primers of Lindström et al. (2001)*

Multiplex PCR was performed to detect BoNT genes in DNA samples with individual BoNT gene types and with all four BoNT gene types (Figure 7). The result showed that multiplex PCR was able to detect the individual BoNT gene in individual gene-containing DNA samples (Lanes 1−6). However, the multiplex PCR was only able to detect two BoNT gene types in DNA sample containing all four BoNT gene types whereas Lindström et al. (2001) were using it to test for all four toxins simultaneously. Also, when a lower concentration of dNTPs were used, as specified by Lindström et al. (2001), only one BoNT gene type could be detected. Given these results, we discontinued using these primers as a multiplex system but used them individually, running a separate lane for each toxin type.

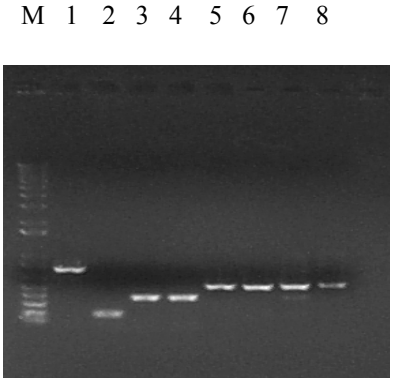

Figure 7: Electrophoresis of PCR products (5 µL) by multiplex PCR amplification of BoNT genes. Lane 1: C. botulinum *Type A; Lane 2:* C. botulinum *Type B17; Lane 3:* C. butyricum *Type E; Lane 4:* C. botulinum *Type E beluga; Lane 5:* C. botulinum *Type F; Lane 6:* C. botulinum *Type 202F; Lane 7: Mixed DNA for all Types; Lane 8: Mixed DNA for all types using the lower concentration of dNTPS specified by Lindström et al. (2001). M: 1 kb DNA ladder (Invitrogen).*

Subsequently (this report, section 9) we used these individual primers to amplify DNA extracted from sediments that had been inoculated with low numbers of spores and enriched to grow the organisms. The amplified PCR products gave clear bands when visualised in agarose gels. An example gel is shown in Figure 8.

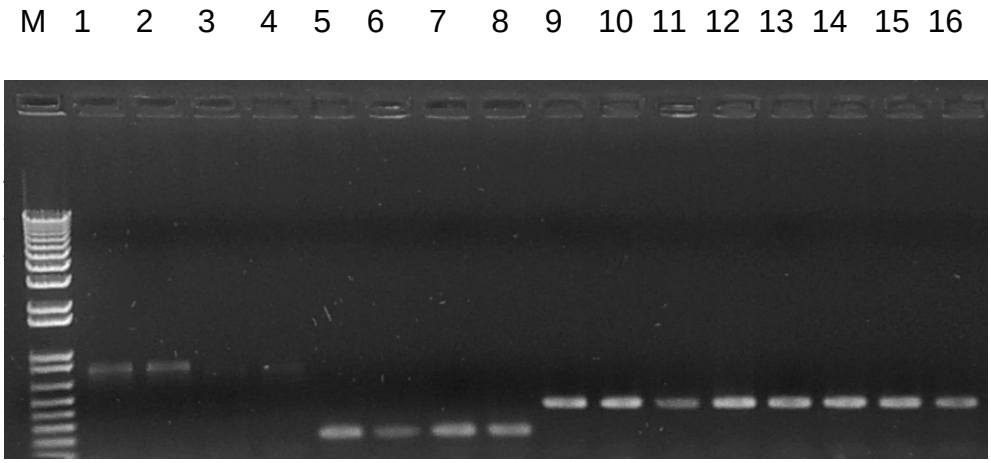

Figure 8: PCR detection of BoNT genes from dilutions of untreated spore-inoculated sediments enriched in TPYG at 30°C (controls Table 11). Lane 1-4: BoNT type A; Lane 5-8: BoNT type 17B; Lane 9-12: BoNT type E Butyricum; Lane 13-16: BoNT type E Beluga. M: 1 kb plus DNA ladder (Invitrogen).

Initially, the sensitivity of the PCR was determined by both diluting spores and extracting DNA from each dilution and by extracting diluting DNA from undiluted spores and diluting the DNA for PCR testing. Results are presented in Table 4 and summarised in Table 5. In some cases the PRC was able to detect toxin from more dilute DNA than from diluted spores. We had hoped that dilution of the DNA subsequent to extraction would give a similar result to dilution of the spores before extraction. This would have allowed the sensitivity of the PCR to be determined with a single DNA extraction rather than extracting each dilution. As this was not the case, in subsequent work we only used a dilution of the spores rather than diluting the DNA to determine the sensitivity of the PCR.

Table 4: Summary of PCR detection for detection of BoNT genes from dilutions of BoNT-producing spores or dilutions of their DNA using the primers of Lindström et al. (2001).

BoNT Type	Dilution type	Spore count/mL	PCR result
A	Spore dilution	7.5×10^{6}	+
A	Spore dilution	7.5×10^{5}	+
A	Spore dilution	7.5×10^{4}	-
A	Spore dilution	7.5×10^{3}	-
A	DNA dilution	10^{-1} of 7.5×10^{6}	+
A	DNA dilution	10^{-2} of 7.5×10^{6}	+[1]
A	DNA dilution	10^{-3} of 7.5×10^{6}	-
A	DNA dilution	10^{-4} of 7.5×10^{6}	-
A	DNA dilution	10^{-5} of 7.5×10^{6}	-
B17	Spore dilution	1.2×10^{7}	+
B17	Spore dilution	1.2×10^{6}	+
B17	Spore dilution	1.2×10^{5}	+
B17	Spore dilution	1.2×10^{4}	-
B17	DNA dilution	10^{-1} of 1.2×10^{7}	+
B17	DNA dilution	10^{-2} of 1.2×10^{7}	+
B17	DNA dilution	10^{-3} of 1.2×10^{7}	+[1]
B17	DNA dilution	10^{-4} of 1.2×10^{7}	-
B17	DNA dilution	10^{-3} of 1.2×10^{7}	-
But-E	Spore dilution	7.5×10^{7}	+
But-E	Spore dilution	7.5×10^{6}	+
But-E	Spore dilution	7.5×10^{5}	+
But-E	Spore dilution	7.5×10^{4}	-
But-E	DNA dilution	$10^{-1}$of 7.5×10^{7}	+
But-E	DNA dilution	$10^{-2}$of 7.5×10^{7}	+
But-E	DNA dilution	$10^{-3}$of 7.5×10^{7}	+
But-E	DNA dilution	$10^{-4}$of 7.5×10^{7}	+[1]
E-Beluga	Spore dilution	6.6×10^{7}	+
E-Beluga	Spore dilution	6.6×10^{6}	+
E-Beluga	Spore dilution	6.6×10^{5}	+
E-Beluga	Spore dilution	6.6×10^{4}	-
E-Beluga	DNA dilution	10^{-1} of 6.6×10^{7}	+
E-Beluga	DNA dilution	10^{-2} of 6.6×10^{7}	+
E-Beluga	DNA dilution	10^{-3} of 6.6×10^{7}	+[1]

BoNT Type	Dilution type	Spore count/mL	PCR result
E-Beluga	DNA dilution	10^{-4} of 6.6 x 10^{7}	-
F	Spore dilution	9.2 x 10^{7}	+
F	Spore dilution	9.2 x 10^{6}	+
F	Spore dilution	9.2 x 10^{5}	+
F	Spore dilution	9.2 x 10^{4}	-
F	Spore dilution	9.2 x 10^{3}	-
F	DNA dilution	10^{-1} of 9.2 x 10^{7}	+
F	DNA dilution	10^{-2} of 9.2 x 10^{7}	+
F	DNA dilution	10^{-3} of 9.2 x 10^{7}	+
F	DNA dilution	10^{-4} of 9.2 x 10^{7}	+[1]
F	DNA dilution	10^{-5} of 9.2 x 10^{7}	-
F202	Spore dilution	4.5 x 10^{6}	+
F202	Spore dilution	4.5 x 10^{5}	+
F202	Spore dilution	4.5 x 10^{4}	+
F202	Spore dilution	4.5 x 10^{3}	-
F202	DNA dilution	10^{-1} of 4.5x10^{6}	+
F202	DNA dilution	10^{-2} of 4.5x10^{6}	+
F202	DNA dilution	10^{-3} of 4.5x10^{6}	+[1]
F202	DNA dilution	10^{-4} of 4.5x10^{6}	-

[1]Dilutions where DNA dilution gave a more sensitive result than was found with spore dilution.

Table 5: Summary of PCR sensitivity for spore detection.

Type	Haemocytometer count (spore/mL)	Sensitivity by spore dilution (spore/mL)	Sensitivity by DNA dilution (spore/mL)
A	7.5×10^7	7.5×10^5	7.5×10^4
B17	1.2×10^8	1.2×10^5	1.2×10^4
But-E	7.5×10^8	7.5×10^5	7.5×10^3
E-Beluga	6.6×10^8	6.6×10^5	6.6×10^4
F	9.2×10^9	9.2×10^5	9.2×10^3
F202	4.5×10^7	4.5×10^4	4.5×10^3

In contrast to the results from spores (Table 1), plate counts and MPNs of vegetative cells were substantially reduced compared with haemocytometer counts (Table 6). This is attributed to the effect of manipulations of vegetative cells under aerobic conditions.

Table 6: Counts of vegetative cells after enrichment of spores (inoculum) for 72 h at 30°C.

Spore strain	Spore inoculum (haemocytometer count cells/mL)	Vegetative cells		
		Plate count (CFU/mL)	MPN/mL	Haemocytometer count (cells/mL)
A	7.50E+04	No growth	2.50E+05	1.30E+08
B17	1.20E+05	No growth	7.78E+04	1.40E+08
But-E	7.5E+05	No growth	3.44E+07	1.60E+08
E-Beluga	6.60E+05	No growth	1.39E+08	1.40E+08
F	9.20E+06	1.7E +06	3.44E+07	1.00E+08
F202	4.50E+04	No growth	2.00E+03	1.20E+08

5.3.3 PCR amplification of BoNT genes using individual primer pairs for vegetative cells

Based on the concentration of vegetative cell by haemocytometry, the sensitivities of PCR detection on individual strains were determined and are summarised in Table 7. The results were generally similar to those of Lindström et al. (2001) who reported a sensitivity of 10^2 cells per reaction mixture for types A, E and F and 10 cells per reaction mixture for type B. The reason for higher detection limits for E-Beluga and F202 toxins might be related to our use of haemocytometer counts for enumeration rather than the blood agar plates used by Lindström et al. (2001).

Table 7: PCR detection limit for vegetative cells.

Strain	Haemocytometer count (cell/mL)	PCR detection limit	
		Cell/mL	Cell/reaction
A	1.3×10^8	1.3×10^4	78
B17	1.4×10^8	1.4×10^3	8
But-E	1.6×10^8	1.6×10^4	96
E-Beluga	1.4×10^8	1.4×10^5	840
F	1.0×10^8	1.0×10^4	60
F202	1.2×10^8	1.2×10^5	720

5.4 Conclusion on selection and amplification of primers

The primers of Lindström et al. are preferred because those of Fach et al. proved not to be specific. However, Lindström et al.'s primers have relatively high detection limits so moderate levels of either the vegetative organisms or higher levels of the spores must be present in the sample before BoNT will be detected. Although the incidence of BoNT in New Zealand is unknown, it is likely to be quite low and, if present, will possibly be present in low numbers in any given sample. To evaluate its incidence it is therefore important that low levels of BoNT-producing organisms can be detected by the PCR. This can be achieved by incubating the samples in an enrichment media suitable for growing the organisms before PCR detection.

The next two sections of this report provide an overview of the literature and describe an experiment to determine suitable enrichment conditions. Section 9 describes an experiment to determine the efficacy of these enrichment procedures followed by PCR to detect low number of BoNT-producing spores in marine sediments.

6 Germination and enrichment conditions • literature

The literature contains varied and sometimes conflicting reports about the best conditions for germinating and growing *C. botulinum* spores.

6.1 Anaerobic media

Two main media have been used to isolate *C. botulinum*: TPYG is recommended by the FDA BAM (Solomon & Lilly 1998), but it contains sodium thioglycollate as a reducing agent, which is toxic and so undesirable for use by laboratory personnel. Other workers (Peck et al. 1992; 1995; Broda et al. 1998a; 1998b) have used PYGS, which is a more complex media and uses cysteine, a benign amino acid, as a reducing agent.

6.2 Incubation temperature

Jensen et al. (1987) looked at the effect of different culture conditions on initiation of growth in media. Growth initiation at 20°C was generally better than initiation at lower temperatures for all strains. Type A had higher probabilities of initiation at 30 than 20°C while growth of Type E-producing spores was similar at both temperatures.

Notermans et al. (1979) noted that *C. botulinum* growth can be inhibited by other *Clostridium* species. They found that, in the presence of *C. botulinum* Type C, the best recovery of Types E and B was at 20°C while Type A could only be recovered when high numbers were added and the best recovery was at 30°C. Types B and E could easily be recovered from low numbers in the presence of competing organisms at 20 (10 spores) and 30°C (100-1000 spores), but not at 37°C.

Bott et al. (1968) found that incubation at 30°C was unsatisfactory for enriching Type E while incubation at 20°C for 14 days or 25°C for 7 days gave good results. Heat treatment (60°C, 30 min) reduced recovery. It was better to test many small samples than a few large samples.

Although sporulation of Type 17B was better at 25 than at 30 or 35°C, sporulation temperature did not affect germination or heat resistance (Peck et al. 1995).

When minced samples were vacuum-packed and incubated at 30°C for 3 days 66.7% of 54 samples were positive (Baker et al. 1990). 33.3% were Type A, 3.7% Type B, 5.6% Type B non-proteolytic and 20.4% Type E. When these samples were also incubated at 12°C for 14 d and 8°C for 14 and 28 days only 29.9, 22.2 and 29.6% respectively of samples were toxic. All fish that became toxic were toxic after incubation at 30°C. However, more toxin Types (Types E and non-proteolytic B) were detected after incubation at lower temperatures.

6.3 Competitive organisms and antimicrobial substances

In a review of Type E toxins, Huss (1981) stated that a negative result is obtained, not only when *C. botulinum* is absent from the specimen, but also when the organism is unable to multiply in the presence of, or in competition with, other micro-organisms. For example, *Bacillus* spp. produce a bacteriocin-like inhibitory substance that inhibits the growth of and toxin production by *C. botulinum* Type E (Lyver et al. 1998). Huss (1981) reviewed work showing that competing organisms produced an antagonistic substance that was digested by tryspin so he proposed including trypsin in enrichment media to overcome antagonistic organisms. Other methods such as grinding with glass powder, heating and ultrasound, have not been found to reduce the effects of competing organisms and antimicrobial substances. However, Huss recommends adding 0.1% trypsin (Difco 250). This made little difference to isolation from marine sediments but in soil and freshwater sediments, trypsin resulted in slightly higher numbers of positive samples and higher toxin titres.

6.4 Pre-treatment with ethanol or heat

The FDA BAM (Solomon & Lilly 1998) states that *C. botulinum* is more readily isolated from mixed flora of enrichment cultures or original specimens if sporulation has been good. They did not recommend pre-treatment with ethanol (EtOH) or heat for non-proteolytic types. However, Broda et al. (1998a) obtained higher rates of recovery of psychrotrophic spore-forming *Clostridia* when ethanol was used to eliminate vegetative competitors.

For proteolytic types heating 1–2 mL of culture or sample at 80°C for 10–15 min is recommended. The optimal heat activation of Type E spores was found to be 60°C for 10 min (Ando & Iida 1970). Huss (1981) reported work that found that heat treatment as mild as 60°C for 30 min did not improve the detection of Type E although heat at 60°C for 15 min is recommended. However, in their sediment samples, heating at 60°C for 10 min did not improve germination of spores (Huss 1981).

EtOH treatment is achieved by adding equal volumes of filter-sterilised absolute EtOH to 1–2 mL enrichment culture in a sterile screw-cap tube. This is mixed well and incubated for 1 h at room temperature. Then 1–2 loopsful of treated cultures are streaked on to liver-veal-egg yolk agar or anaerobic egg yolk agar. Plates are then dried and incubated under anaerobic conditions. However, treatment of samples with 50% EtOH had limited success (Huss 1981). Growing Types A and B in TPYG with added EtOH (1, 2, 3 or 4%) reduced growth rates and increased lag phases (Chea et al. 2000). Where TPYG with EtOH tubes did not show any growth after 1 year at 25°C, sub-culturing to TPYG invariably enhanced growth, i.e. EtOH inhibited germination but did not affect viability.

6.5 Media composition, germinants, etc.

Braconnier et al. (2001) found that addition of a germinant mixture (L-cysteine, L-alanine, L-lactate to final respective concentrations of 10, 50 and 50 mM) sometimes gave a marked increase in germination of Types A and B proteolytic strains in various media. Germination of Type E required L-alanine, glucose, lactate and bicarbonate and bacto-casitone was found to be a better peptone for germination that the other peptones tested (Ando & Iida 1970). Broussolle et al. (2002) found that germination was strongly triggered by L-alanine/L-lactate/$NaHCO_3$ (20 mM/10 mM/100 mM) in phosphate buffer (pH 7). Spores were first heat shocked, treated with lysozyme (0.2 mg/mL) at 37°C for 10 min then mixed with the germinant mixture. Optimal germination of Type E was recorded as occurring in a mixture of L-alanine (100 mM), L-lactate (50 mM), $NaHCO_3$ (50 mM) and 25 mM sodium thioglycollate in 100 mM phosphate buffer containing sterile salmon extract (Rutherford et al. 2003). Peck et al. (1992) found that heat-treated spores were better recovered on media incorporating lysozyme (PYGS + 10 µg/mL lysozyme) after pre-treatment with agents that increase spore permeability (e.g. alkaline thioglycolate). They surmised that the heat treatment had inactivated the germination system, which could be recovered by the presence of lysozyme. The permeation agent increased the ability of lysozyme to enter the spore.

7 Enrichment media and incubation temperature

7.1 Method

1. A set of 4-well MPNs were performed for each strain in PYGS and TPYG broths.
2. The original spores suspensions (Table 1) were diluted to counts of between 10^5 and 10^6 spore/L and the MPNs performed on eight serial dilutions of these.
3. A set of 4 well MPN plates (6 strains x 2 broths; PYGS or TPYG) was incubated at 15, 20, 25, 30 and 35°C.
4. Also, to test the usefulness of changing the media composition as discussed in section 6.5 above, two more sets of 4-well MPNs for each strain using TPYG containing L-alanine and L-lactate at final concentrations of 100 and 50 mM (TPYG+Al,Lac) and TPYG containing 10 µg/mL lysozyme (TPYG+Lys) were carried out and incubated at 25°C.
5. The plates were read by recording turbidity (620 nm) in each well using the Multiskan Ex (Thermo Electronic Co-operation) Plate Reader after 1, 2, 3, 4, 5, 6, 7, 10, 14, 21 and 35 days. Recoveries were calculated

based on the estimates (Table 1) of the original concentrations of spores for the BoNT-producing types:

8. A 7.5 x 10^5 spore/mL
9. B17 1.2 x 10^5 spore/mL
10. but-E 7.6 x 10^5 spore/mL
11. E-beluga 6.6 x 10^5 spore/mL
12. F 9.2 x 10^5 spore/mL
13. F202 4.5 x 10^5 spore/mL
14.

Haldane's Approximation to the standard errors of the $\log_{10}$ MPNs as generated by the BAM-MPN Calculator (www.cfsan.fda.gov/~ebam/bam-a2.html#excl) were used to compare results. Results for which standard errors did not overlap were considered to be significantly different from each other. The criteria for selecting the best media and temperature were, in order of priority, the ones giving:

- The highest MPNs (most sensitive);
- The fastest growth rates (most likely to compete against competitive microflora).

7.2 Results

All results after 4, 14 and 35 days' incubation are presented in Tables 8-10. The TPYG data from temperatures between 25 and 35°C are presented graphically in Figures 9-11.

*Table 8: Counts, detection and recovery of different BoNT-*producing *strains in different media after 4 days' incubation at different temperatures.*

Media and temperature	Strain	$\log_{10}$ MPN	$\log_{10}$ MPN se	Recovery (%)	Mean overall recovery across strains (%)
TPYG, 15°C	A	<1.10		0.00	
TPYG, 15°C	B17	<1.10		0.00	
TPYG, 15°C	But-E	<1.10		0.00	
TPYG, 15°C	E-Beluga	<1.10		0.00	
TPYG, 15°C	F	<1.10		0.00	
TPYG, 15°C	F202	<1.10		0.00	0.00
PYGS, 15°C	A	<1.10		0.00	
PYGS, 15°C	B17	<1.10		0.00	
PYGS, 15°C	But-E	<1.10		0.00	
PYGS, 15°C	E-Beluga	<1.10		0.00	
PYGS, 15°C	F	<1.10		0.00	

Media and temperature	Strain	Log_{10} MPN	Log_{10} MPN se	Recovery (%)	Mean overall recovery across strains (%)
PYGS, 15°C	F202	<1.10		0.00	0.00
TPYG, 20°C	A	2.11	0.27	0.02	
TPYG, 20°C	B17	3.19	0.20	1.28	
TPYG, 20°C	But-E	4.53	0.29	4.54	
TPYG, 20°C	E-Beluga	2.36	0.18	0.03	
TPYG, 20°C	F	2.11	0.27	0.01	
TPYG, 20°C	F202	1.71	0.26	0.01	0.98
PYGS, 20°C	A	<1.10		0.00	
PYGS, 20°C	B17	<1.10		0.00	
PYGS, 20°C	But-E	<1.10		0.00	
PYGS, 20°C	E-Beluga	<1.10		0.00	
PYGS, 20°C	F	<1.10		0.00	
PYGS, 20°C	F202	<1.10		0.00	0.00
TPYG, 25°C	A	5.86	0.23	97.54	
TPYG, 25°C	B17	5.27	0.27	156.46	
TPYG, 25°C	But-E	4.70	0.25	6.75	
TPYG, 25°C	E-Beluga	4.30	0.27	3.01	
TPYG, 25°C	F	5.86	0.23	79.51	
TPYG, 25°C	F202	5.86	0.23	162.56	84.31
PYGS, 25°C	A	4.79	0.26	8.24	
PYGS, 25°C	B17	1.15	0.44	0.01	
PYGS, 25°C	But-E	<1.10		0.00	
PYGS, 25°C	E-Beluga	1.52	0.31	0.01	
PYGS, 25°C	F	5.98	0.24	103.72	
PYGS, 25°C	F202	2.11	0.27	0.03	18.67
TPYG+Al,Lac, 25°C	A	5.27	0.27	25.02	
TPYG+Al,Lac, 25°C	B17	3.11	0.27	1.07	
TPYG+Al,Lac, 25°C	But-E	1.80	0.26	0.01	
TPYG+Al,Lac, 25°C	E-Beluga	1.52	0.31	0.01	
TPYG+Al,Lac, 25°C	F	6.10	0.23	138.09	
TPYG+Al,Lac, 25°C	F202	5.10	0.27	28.03	32.04
TPYG+Lys, 25°C	A	5.74	0.26	73.31	
TPYG+Lys, 25°C	B17	4.79	0.26	51.48	
TPYG+Lys, 25°C	But-E	3.95	0.23	1.18	
TPYG+Lys, 25°C	E-Beluga	2.80	0.26	0.09	

Media and temperature	Strain	Log_{10} MPN	Log_{10} MPN se	Recovery (%)	Mean overall recovery across strains (%)
TPYG+Lys, 25°C	F	6.10	0.23	138.09	
TPYG+Lys, 25°C	F202	5.27	0.27	41.70	50.98
TPYG, 30°C	A	6.23	0.22	227.42	
TPYG, 30°C	B17	5.27	0.27	156.38	
TPYG, 30°C	But-E	4.11	0.27	1.70	
TPYG, 30°C	E-Beluga	4.79	0.26	9.36	
TPYG, 30°C	F	6.10	0.23	138.09	
TPYG, 30°C	F202	5.98	0.24	211.21	124.03
PYGS, 30°C	A	4.95	0.23	11.77	
PYGS, 30°C	B17	3.30	0.28	1.66	
PYGS, 30°C	But-E	1.52	0.31	0.00	
PYGS, 30°C	E-Beluga	<1.10		0.00	
PYGS, 30°C	F	4.53	0.29	3.69	
PYGS, 30°C	F202	2.53	0.29	0.08	2.87
TPYG, 35°C	A	6.10	0.23	169.40	
TPYG, 35°C	B17	2.95	0.23	0.74	
TPYG, 35°C	But-E	4.53	0.29	4.53	
TPYG, 35°C	E-Beluga	4.30	0.27	3.01	
TPYG, 35°C	F	6.52	0.21	357.98	
TPYG, 35°C	F202	5.74	0.26	122.19	109.64
PYGS, 35°C	A	4.30	0.27	2.65	
PYGS, 35°C	B17	3.30	0.28	1.66	
PYGS, 35°C	But-E	1.15	0.44	0.00	
PYGS, 35°C	E-Beluga	<1.10		0.00	
PYGS, 35°C	F	4.79	0.26	6.71	
PYGS, 35°C	F202	2.11	0.27	0.03	1.84

Table 9: Counts and percentage recovery of different BoNT-producing strains in TPYG after 14 days' incubation at different temperatures.

Media and temperature	Strain	Log_{10} MPN	Log_{10} MPN s.e.	Recovery (%)	Mean overall recovery across strains (%)
TPYG, 15°C	A	3.95	0.23	1.18	
TPYG, 15°C	B17	3.65	0.25	3.69	
TPYG, 15°C	But-E	3.53	0.29	0.45	
TPYG, 15°C	E-beluga	3.17	0.20	0.23	
TPYG, 15°C	F	5.98	0.24	103.31	
TPYG, 15°C	F202	2.17	0.20	0.03	18.15
PYGS, 15°C	A	<1.10		0.00	
PYGS, 15°C	B17	<1.10		0.00	
PYGS, 15°C	But-E	1.52	0.31	0.00	
PYGS, 15°C	E-beluga	2.11	0.27	0.02	
PYGS, 15°C	F	<1.10		0.00	
PYGS, 15°C	F202	1.80	0.26	0.01	0.01
TPYG, 20°C	A	6.23	0.22	227.42	
TPYG, 20°C	B17	3.80	0.26	5.20	
TPYG, 20°C	But-E	4.53	0.29	4.53	
TPYG, 20°C	E-beluga	3.80	0.26	0.95	
TPYG, 20°C	F	5.98	0.24	103.31	
TPYG, 20°C	F202	2.71	0.26	0.11	56.92
PYGS, 20°C	A	4.79	0.26	8.24	
PYGS, 20°C	B17	2.11	0.27	0.11	
PYGS, 20°C	But-E	2.11	0.27	0.02	
PYGS, 20°C	E-beluga	1.80	0.26	0.01	
PYGS, 20°C	F	5.27	0.27	20.40	
PYGS, 20°C	F202	2.11	0.27	0.03	4.80
TPYG, 25°C	A	5.86	0.23	97.54	
TPYG, 25°C	B17	5.27	0.27	156.47	
TPYG, 25°C	But-E	4.70	0.25	6.75	
TPYG, 25°C	E-beluga	4.30	0.27	3.01	
TPYG, 25°C	F	5.86	0.23	79.51	
TPYG, 25°C	F202	5.86	0.23	162.56	84.31
PYGS, 25°C	A	5.86	0.23	97.54	
PYGS, 25°C	B17	3.71	0.26	4.29	

Media and temperature	Strain	Log_{10} MPN	Log_{10} MPN s.e.	Recovery (%)	Mean overall recovery across strains (%)
PYGS, 25°C	But-E	<1.10		0.00	
PYGS, 25°C	E-beluga	1.80	0.26	0.01	
PYGS, 25°C	F	5.98	0.24	103.72	
PYGS, 25°C	F202	2.11	0.27	0.03	34.26
TPYG+Al,Lac, 25°C	A	6.23	0.22	227.42	
TPYG+Al,Lac, 25°C	B17	3.11	0.27	1.07	
TPYG+Al,Lac, 25°C	But-E	1.80	0.26	0.01	
TPYG+Al,Lac, 25°C	E-beluga	1.52	0.31	0.01	
TPYG+Al,Lac, 25°C	F	6.10	0.23	138.10	
TPYG+Al,Lac, 25°C	F202	5.27	0.27	41.70	68.05
TPYG+Lys, 25°C	A	5.98	0.24	127.23	
TPYG+Lys, 25°C	B17	4.79	0.26	51.48	
TPYG+Lys, 25°C	But-E	3.95	0.23	1.18	
TPYG+Lys, 25°C	E-beluga	2.80	0.26	0.09	
TPYG+Lys, 25°C	F	6.10	0.23	138.10	
TPYG+Lys, 25°C	F202	5.27	0.27	41.70	59.96
TPYG, 30°C	A	6.23	0.22	227.42	
TPYG, 30°C	B17	5.27	0.27	156.38	
TPYG, 30°C	But-E	4.11	0.27	1.70	
TPYG, 30°C	E-beluga	4.79	0.26	9.36	
TPYG, 30°C	F	6.10	0.23	138.10	
TPYG, 30°C	F202	5.98	0.24	211.21	124.03
PYGS, 30°C	A	5.50	0.28	42.47	
PYGS, 30°C	B17	3.80	0.26	5.20	
PYGS, 30°C	But-E	1.52	0.31	0.00	
PYGS, 30°C	E-beluga	1.15	0.44	0.00	
PYGS, 30°C	F	5.50	0.28	34.62	
PYGS, 30°C	F202	2.53	0.29	0.08	13.73
TPYG, 35°C	A	6.10	0.23	169.40	
TPYG, 35°C	B17	4.11	0.27	10.63	
TPYG, 35°C	But-E	4.79	0.26	8.24	
TPYG, 35°C	E-beluga	5.07	0.21	17.79	
TPYG, 35°C	F	6.52	0.21	357.98	
TPYG, 35°C	F202	5.74	0.26	122.19	114.37
PYGS, 35°C	A	4.30	0.27	2.65	

Media and temperature	Strain	Log_{10} MPN	Log_{10} MPN s.e.	Recovery (%)	Mean overall recovery across strains (%)
PYGS, 35°C					
PYGS, 35°C	B17	3.53	0.29	2.84	
PYGS, 35°C	But-E	1.15	0.44	0.00	
PYGS, 35°C	E-beluga	<1.10		0.00	
PYGS, 35°C	F	5.10	0.27	13.71	
PYGS, 35°C	F202	2.11	0.27	0.03	3.21

*Table 10: Counts and percentage recovery of different BoNT-*producing *strains in different media after 35 days' at different temperatures.*

Media and temperature	Strain	Log_{10} MPN	Log_{10} MPN se	Recovery (%)	Mean overall recovery across strains (%)
TPYG, 15°C	A	5.27	0.27	25.02	
TPYG, 15°C	B17	3.65	0.25	3.69	
TPYG, 15°C	But-E	3.95	0.23	1.18	
TPYG, 15°C	E-beluga	3.17	0.20	0.23	
TPYG, 15°C	F	5.98	0.24	103.31	
TPYG, 15°C	F202	2.17	0.20	0.03	22.24
PYGS, 15°C	A	<1.10		0.00	
PYGS, 15°C	B17	1.15	0.44	0.01	
PYGS, 15°C	But-E	1.52	0.31	0.00	
PYGS, 15°C	E-beluga	2.11	0.27	0.02	
PYGS, 15°C	F	<1.10		0.00	
PYGS, 15°C	F202	1.80	0.26	0.01	0.01
TPYG, 20°C	A	6.23	0.22	227.42	
TPYG, 20°C	B17	3.80	0.26	5.20	
TPYG, 20°C	But-E	4.53	0.29	4.53	
TPYG, 20°C	E-beluga	3.80	0.26	0.95	
TPYG, 20°C	F	5.98	0.24	103.31	
TPYG, 20°C	F202	3.65	0.25	0.98	57.06
PYGS, 20°C	A	4.79	0.26	8.24	
PYGS, 20°C	B17	3.65	0.25	3.69	
PYGS, 20°C	But-E	2.11	0.27	0.02	

Media and temperature	Strain	Log_{10} MPN	Log_{10} MPN se	Recovery (%)	Mean overall recovery across strains (%)
PYGS, 20°C	E-beluga	1.80	0.26	0.01	
PYGS, 20°C	F	5.27	0.27	20.40	
PYGS, 20°C	F202	2.11	0.27	0.03	5.40
TPYG, 25°C	A	5.86	0.23	97.54	
TPYG, 25°C	B17	5.27	0.27	156.46	
TPYG, 25°C	But-E	4.70	0.25	6.75	
TPYG, 25°C	E-beluga	4.30	0.27	3.01	
TPYG, 25°C	F	5.86	0.23	79.51	
TPYG, 25°C	F202	5.86	0.23	162.56	84.31c
PYGS, 25°C	A	5.86	0.23	97.54	
PYGS, 25°C	B17	3.71	0.26	4.29	
PYGS, 25°C	But-E	<1.10		0.00	
PYGS, 25°C	E-beluga	1.80	0.26	0.01	
PYGS, 25°C	F	5.98	0.24	103.72	
PYGS, 25°C	F202	2.11	0.27	0.03	34.26
TPYG+Al,Lac, 25°C	A	6.23	0.22	227.42	
TPYG+Al,Lac, 25°C	B17	3.65	0.25	3.69	
TPYG+Al,Lac, 25°C	But-E	1.80	0.26	0.01	
TPYG+Al,Lac, 25°C	E-beluga	1.52	0.31	0.01	
TPYG+Al,Lac, 25°C	F	6.10	0.23	138.09	
TPYG+Al,Lac, 25°C	F202	5.27	0.27	41.70	68.49
TPYG+Lys, 25°C	A	5.98	0.24	127.23	
TPYG+Lys, 25°C	B17	4.79	0.26	51.48	
TPYG+Lys, 25°C	But-E	3.95	0.23	1.18	
TPYG+Lys, 25°C	E-beluga	2.80	0.26	0.09	
TPYG+Lys, 25°C	F	6.10	0.23	138.09	
TPYG+Lys, 25°C	F202	5.27	0.27	41.70	59.96
TPYG, 30°C	A	6.23	0.22	227.42	
TPYG, 30°C	B17	5.27	0.27	156.38	
TPYG, 30°C	But-E	4.11	0.27	1.70	
TPYG, 30°C	E-beluga	4.79	0.26	9.36	
TPYG, 30°C	F	6.10	0.23	138.09	
TPYG, 30°C	F202	5.98	0.24	211.21	124.03
PYGS, 30°C	A	5.50	0.28	42.47	
PYGS, 30°C	B17	4.11	0.27	10.63	

Media and temperature	Strain	Log_{10} MPN	Log_{10} MPN se	Recovery (%)	Mean overall recovery across strains (%)
PYGS, 30°C	But-E	1.80	0.26	0.01	
PYGS, 30°C	E-beluga	1.15	0.44	0.00	
PYGS, 30°C	F	5.50	0.28	34.62	
PYGS, 30°C	F202	2.53	0.29	0.08	14.63
TPYG, 35°C	A	6.10	0.23	169.40	
TPYG, 35°C	B17	4.79	0.26	51.48	
TPYG, 35°C	But-E	4.79	0.26	8.24	
TPYG, 35°C	E-beluga	5.07	0.21	17.79	
TPYG, 35°C	F	6.52	0.21	357.98	
TPYG, 35°C	F202	5.98	0.24	211.21	136.02
PYGS, 35°C	A	4.30	0.27	2.65	
PYGS, 35°C	B17	3.53	0.29	2.84	
PYGS, 35°C	But-E	1.15	0.44	0.00	
PYGS, 35°C	E-beluga	<1.10		0.00	
PYGS, 35°C	F	5.10	0.27	13.71	
PYGS, 35°C	F202	2.11	0.27	0.03	3.21

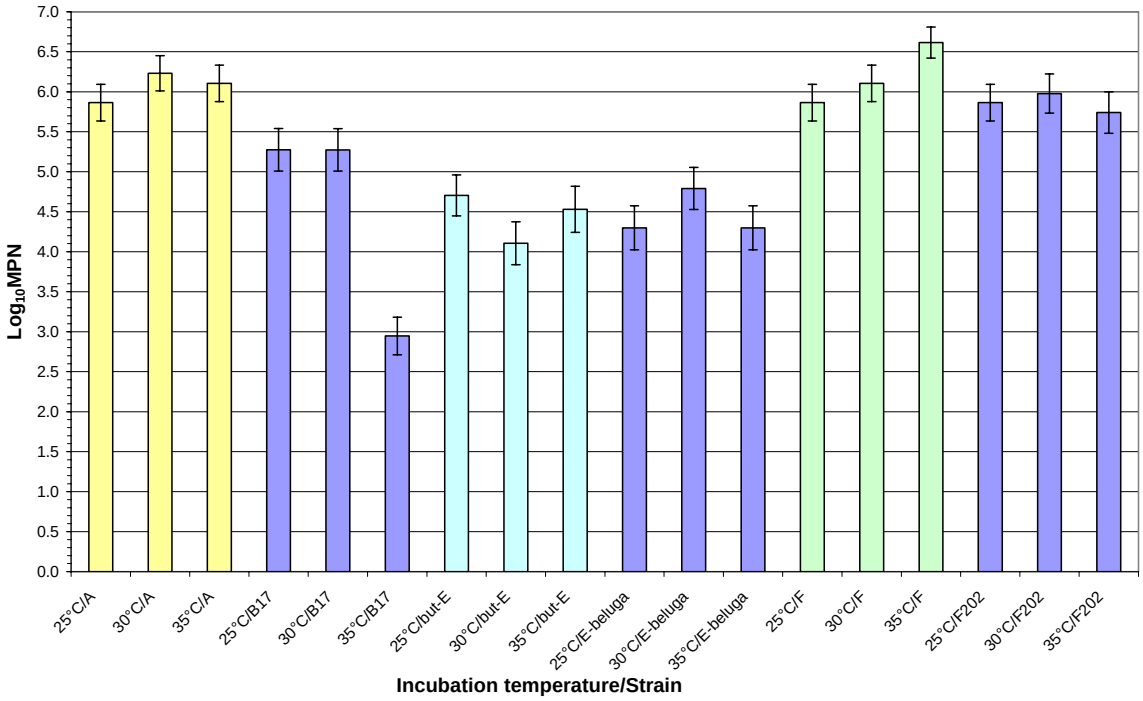

Figure 9: Counts after 4 days' incubation in TPYG broth at 25, 30 & 35°C. Error bars represent standard errors of the log_{10} MPNs.

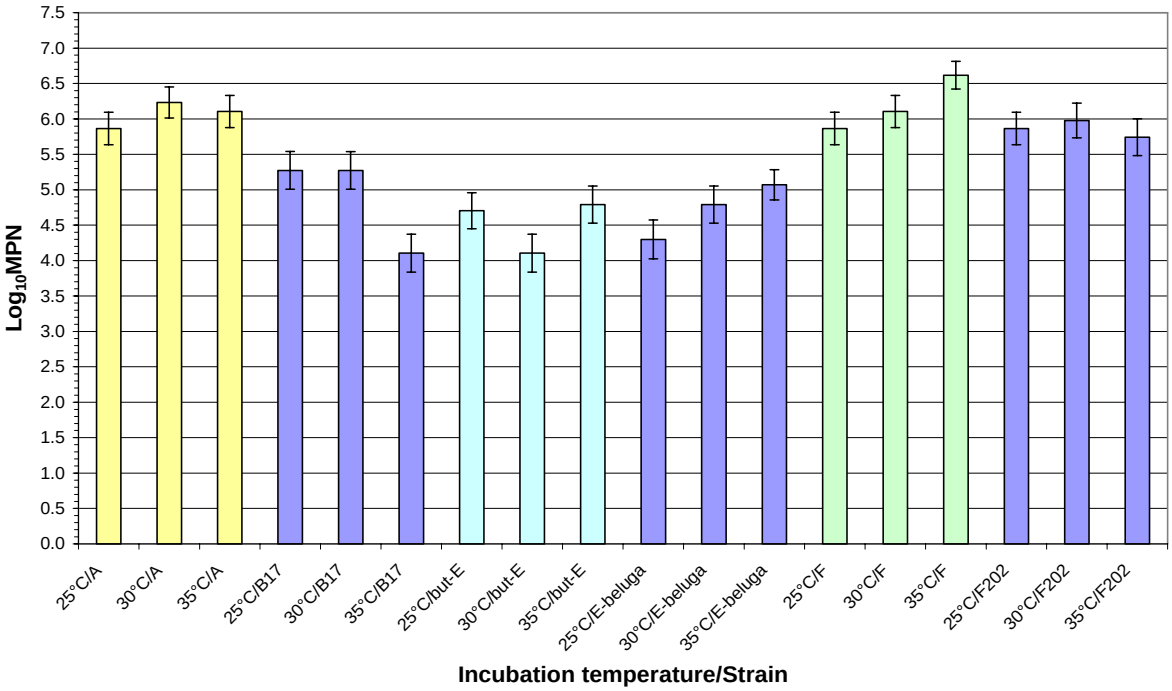

Figure 10: Counts after 14 days' incubation in TPYG broth at 25, 30 & 35°C. Error bars represent standard errors of the log_{10} MPNs.

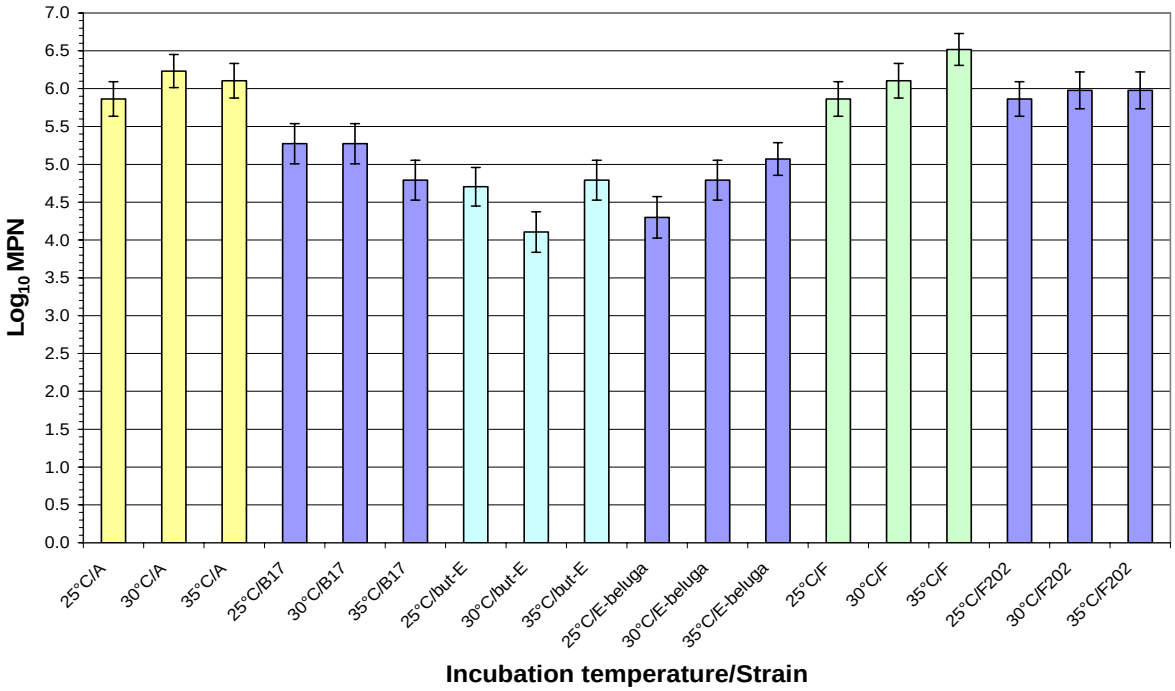

Figure 11: Counts after 35 days' incubation in TPYG broth at 25, 30 & 35°C. Error bars represent standard errors of the log_{10} MPNs.

7.3 *Conclusions from enrichment of spores in broths*

- TPYG consistently outperformed PYGS both in terms of sensitivity and growth rate. This result may be related to culture manipulations being carried out in aerobic conditions rather than an anaerobic chamber. Solomon & Lilly (1998) define TPYG using steaming to eliminate oxygen from the media and allow for aerobic manipulations of samples. From the recovery results, TYPG seems to produce good results from spores under such conditions. Groups using PYGS typically carry out all manipulations in an anaerobic chamber and cool the media under an anaerobic gas flow to ensure that it remains oxygen-free as specified in the original reference for this media (Lund et al. 1990). TPYG rather than PYGS should be used by groups using anaerobic jars rather than anaerobic cabinets.
- Including alanine/lactate or lysozyme in the TPYG medium did not enhance the sensitivity or growth rate.
- Sensitivity and growth rates were substantially lower when incubated at 15 and 20°C than when incubated at higher temperatures.
- But-E had lower counts at 30°C than at 25 or 35°C, but this is probably an aberration in that these counts were also lower than those that at 20°C and the counts in the PYGS did not follow the same pattern.
- Counts did not increase between 14 and 35 days' incubation except for B17, which continued to grow slowly at 35°C.

At 14 days' incubation:

- At 35°C, B17, one of the strains of particular interest for New Zealand marine sediments had a significantly lower MPN than at 30 or 25°C;
- E-beluga and F had significantly lower MPNs at 20°C than at 35°C;
- These results indicate that 14 days' incubation in TPYG at 30°C is optimal for growth and recovery of all strains of BoNT-producing organisms.

8 Overcoming sediment and competitive flora effects

Although experiments carried out on pure cultures in enrichment media can be used to identify optimum germination and growth conditions, they do not necessarily reflect what will happen in real samples where competitive microflora are present and where the composition of the sample may affect bacterial growth. The following experiment was therefore carried out. Various numbers of spores were added to untreated marine sediment collected from an area previously tested as negative for BoNT, along with the selected enrichment media (TPYG) followed by incubation (2–5 weeks at 30°C). The ability of the PCR method to detect the BoNT gene was then determined. This closely reflects the methods that we proposed be used for unknown marine samples.

8.1 Method

Three tube MPNs were prepared using the selected base medium (TPYG broth) as follows:

1. 1 g sediment was added to 12.5 mL tubes.
2. The six BoNT-producing spore crops were diluted such that a 0.1 mL aliquot contained just more than 10 times the minimum number of spores detectable by the PCR (10^6).
3. Decimal dilutions of the above were carried out to a level (last dilution) calculated to contain just less than 1 spore/0.1 mL.
4. 0.1 mL of each dilution of each strain was then dispensed to three tubes of sediment for each of four lots.
5. 9 mL of TPYG broth was added to each tube of the first lot (Control).
6. Each tube of the second lot (EtOH) was mixed with 5 mL ethanol (50%), held 60 min at 20°C, then centrifuged (15 min at 500 rpm) and the ethanol discarded. Then, 9 mL of TPYG broth was added to each tube.
7. Each tube of the third lot (Heat) was heated (60°C, 10 min), rapidly cooled to 4°C, then 9 mL of TPYG broth was added to each tube.
8. Filter sterilised trypsin was added to the TPYG broth (final concentration 0.1%) and 9 mL added to each tube for the fourth lot (Trypsin).
9. All tubes of the four lots were incubated anaerobically at 30°C.
10. After 14 days' incubation at 30°C, 1 mL from each tube was removed and stored frozen at -85°C.
11. After 35 days' incubation the MPN tubes were frozen at -85°C.
12. Tubes (at least 9) from the aliquots of each BoNT-producing strain that had been frozen after 14 days' incubation were selected for PCR testing

for the BoNT genes using single or multiple primers. Tubes of various dilutions were tested until sufficient positive and negative tubes were found to perform a 3-tube 3-dilution MPN determination as follows: the sediment cultures with enriched spores from different dilutions were mixed thoroughly by vortexing. Samples of 30 µL of culture were taken from each dilution for DNA extraction by FastPrep® bead-beating as described previously. Two µL from each DNA sample was used for PCR amplification. Ten µL of PCR product was loaded for electrophoresis analysis for the presence of the BoNT genes in the inoculated sediment cultures. These results were compared with those from the 1 mL aliquots from samples enriched for 35 days to determine how long samples needed to be enriched in the presence of sediment. Results for which standard errors did not overlap were considered to be significantly different from each other.

8.2 Results

Table 11 summarises the highest dilutions giving positive PCR results for single and multiple primers from the four enrichment and pre-enrichment treatments. The multiplex primer system of Lindström et al. (2001) was less sensitive than when the same primers were used singularly. Only a few tubes were tested at 5 weeks' incubation. Incubation for 5 weeks did not appear to improve sensitivity except for Type A. Incubation for 5 weeks from the control treatment apparently decreased the recovery of Type F202.

Counts of spores before and after 14 days' enrichment are presented in Table 12 and Figure 12. The EtOH pre-enrichment treatment resulted in the lowest recovery of spores and significantly reduced recovery from the psychrotrophic strains (B17, E-beluga, F202) and from But-E. For the remaining methods:

- The control gave better recovery for Type F202.
- Heat treatment before enrichment gave better recovery of Type A.
- Recovery of the other strains was not significantly affected by the treatments.

Table 11: Summary PCR detection[1] of BoNT genes in enriched sediments

Type	Treatment	Spores in the first tube	By multiple primers at week 2			By single primers at week 2			Replicate 1 at week 5	Replicate 1 at week 5
			Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	multiple primers	single primers
A	Control	7.5×10^6	3	<2	3	3	4	4	<4	4
	Ethanol		2	<2	<2	3	4	4	<4	5[2]
	Heat		4	<3	<2	4	5	4	<4	5
	Trypsin		<2	<2	<2	3	4	4	<4	5[2]
B17	Control	1.2×10^6	8	6	7	8	7	7	6	ND
	Ethanol		<2	<2	<4	4	3	4	4	4
	Heat		7	7	8	7	7	8	7	ND
	Trypsin		6	7	6	7	7	8	7	ND
But-E	Control	7.5×10^6	6	6	6	6	6	6	6	ND
	Ethanol		<1	<1	<1	4	4	4	<3	<3
	Heat		6	<6	<6	6	6	7	6	ND
	Trypsin		6	6	7	6	6	7	6	ND
E-Beluga	Control	6.6×10^6	5	5	4	6	5	5	<6	<6
	Ethanol		<1	2	<1	<1	1	<1	<3	<3
	Heat		6	<5	<5	6	6	6	6	ND
	Trypsin		6	<4	6	6	5	6	6	ND
F	Control	9.2×10^6	5	6	5	5	6	6	<6	<6
	Ethanol		5	5	5	5	5	5	<6	<6
	Heat		5	4	<5	6	5	6	5	ND
	Trypsin		4	5	5	6	5	6	<5	5
F202	Control	4.5×10^6	7	4	6	7	7	7	<5	<5[3]
	Ethanol		4	<3	<3	4	<3	<3	<4	<4
	Heat		5	6	5	5	6	5	<6	<6
	Trypsin		4	<4	<4	6	4	<4	<5	<5

[1] Data represent highest log dilutions where PCR detected positive.
[2] Detection in more dilute tube at 5 weeks compared to any replicate at 2 weeks.
[3] Detection in more dilute tube at 2 weeks compared to 5 weeks' incubation.
< means PCR negative at this dilution and no DNA sample tested at a lower dilution.
ND = not tested.

Table 12: PCR detected MPNs and detection limits for BoNT gene for sediments inoculated with six different botulinum strains, enriched for 14 days in TPGY broth and incubated at 30°C.

		Log10 spores/tube*				Spores/tube*			
Treatment	Strain	Added spores	Detected MPN	Detection SE	Detection limit (log)	Detection limit	Detection lower 95% CI	Detection upper 95% CI	Mean recovery across strains (%)
Control	A	6.88	4.70	0.32	2.18	150	70	320	121.71
Control	B17	6.08	6.77	0.17	-0.69	0.2	0.14	0.3	
Control	But-E	6.88	6.52	0.24	0.36	2.3	1.3	4	
Control	E-beluga	6.82	6.15	0.26	0.67	4.7	2.6	8.5	
Control	F	6.96	6.33	0.25	0.63	4.3	2.4	7.6	
Control	F202	6.65	6.83	0.18	-0.18	0.66	0.44	1	
EtOH	A	6.88	4.70	0.32	2.18	150	70	320	2.84
EtOH	B17	6.08	4.70	0.32	1.38	24	11	50	
EtOH	But-E	6.88	5.10	0.31	1.78	60	30	123	
EtOH	E-beluga	6.82	1.30	0.44	5.52	330000	120000	910000	
EtOH	F	6.96	5.98	0.28	0.98	9.5	5	18.2	
EtOH	F202	6.65	3.61	0.31	3.04	1100	500	2200	
Heat	A	6.88	5.33	0.31	1.55	35	17	72	109.44
Heat	B17	6.08	6.77	0.17	-0.69	0.2	0.14	0.3	
Heat	But-E	6.88	6.65	0.22	0.23	1.7	1	2.8	
Heat	E-beluga	6.82	6.52	0.24	0.30	2	1.1	3.5	
Heat	F	6.96	6.33	0.25	0.63	4.3	2.4	7.6	
Heat	F202	6.65	6.15	0.26	0.50	3.2	1.7	5.8	
Trypsin	A	6.88	4.70	0.32	2.18	150	70	320	101.26
Trypsin	B17	6.08	6.77	0.17	-0.69	0.2	0.14	0.3	
Trypsin	But-E	6.88	6.65	0.22	0.23	1.7	1	2.8	
Trypsin	E-beluga	6.82	6.33	0.25	0.49	3.1	1.7	5.5	
Trypsin	F	6.96	6.33	0.25	0.63	4.3	2.4	7.6	
Trypsin	F202	6.65	5.04	0.23	1.61	41	24	69	

*Each tube contained 1 g sediment and 9 mL of TPYG broth.

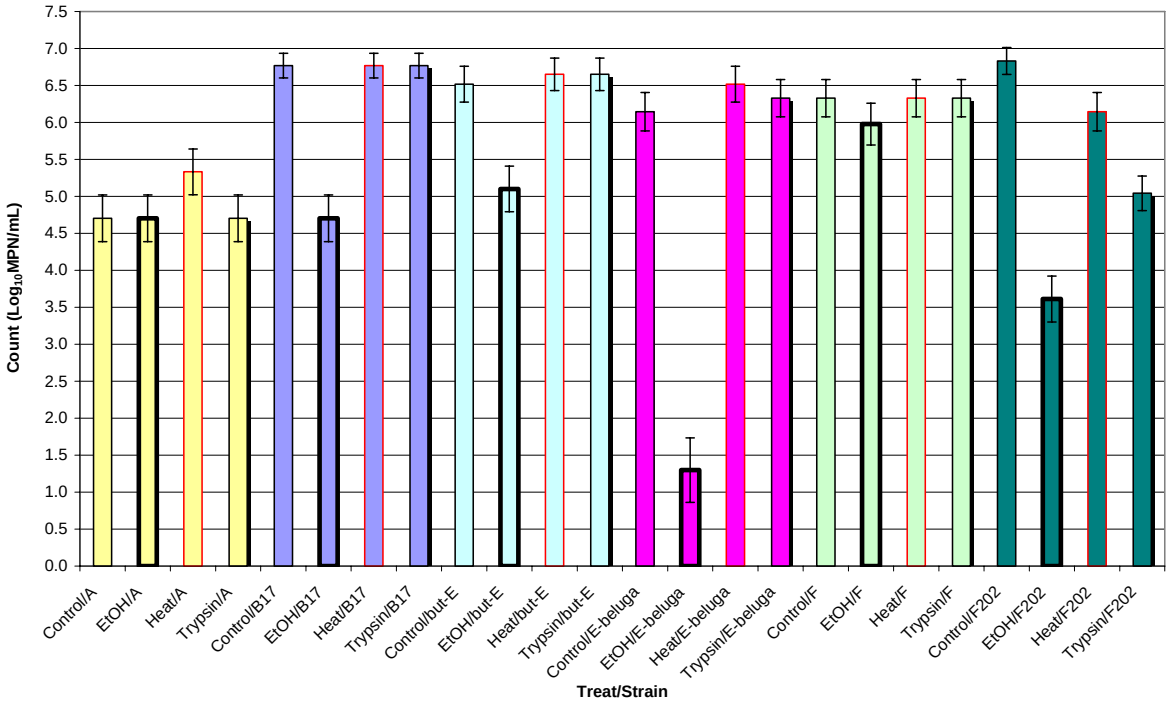

Figure 12: Log_{10} MPNs of BoNT gene detected in sediments inoculated with six different botulinum strains, enriched in TPGY broth and incubated at 30°C for 14 days. Error bars represent standard errors of the log_{10} MPNs.

8.3 Conclusions from enrichment of sediments

This experiment provides a realistic evaluation of the actual test method we plan to use for sediment samples containing unknown levels of BoNT-producing organisms. The main difference is that in this experiment we tested 1 g of sediment in 9 mL of enrichment media whereas in the proposed actual test method we will test 10 g sediment in 90 mL media. The sediment used in this experiment was a real sample with its natural microflora still intact. However, different sediments would have different microflora so this represents a source of uncertainty affecting the sensitivity of the assay when applied to unknown sediments.

Although there were few differences between the treatments, because of its better recovery for Type F202, the control treatment (no pre-enrichment treatment and enrichment without trypsin) was the best overall enrichment strategy. We propose that this procedure is therefore implemented when testing unknown sediment samples.

In the control treatment, all strains were recovered at starting levels of less than five spores per tube after 14 days' incubation except for Type A, which could only be recovered when at least 150 spores were present per tube. Type A is not usually recovered from the marine environment but it is important to the current study as it is the only human toxin type known to be present in New Zealand (Flacks 1985). Heat treatment before enrichment improved the recovery of Type A with a detection limit of 35 spores so we propose to use the heat treatment on a second 10 g sample as well as the control treatment on 10 g of sample. Incubation for 5 weeks rather than just

2 weeks also increased the recovery of Type A. In contrast, recovery of Type F202 decreased during this storage period. Thus to detect low numbers of BoNT-producing spores in sediment, cultures will be tested by PCR after both 14 and 35 days' incubation at 30°C. Results of pure cultures also indicate benefits from testing after 5 weeks' incubation. In pure cultures, detection of BoNT-producing *Clostridium* did not change during enrichment in TPYG at 30°C from 14 to 35 days but Type B17 increased over this time period when enriched in TPYG at 35°C (Tables 9 and 10). Degradation of F202 DNA in sediment cultures but not in pure cultures suggests that competitive flora could be feeding on or otherwise degrading these organisms.

As results from this section indicated that best recovery sometimes occurred after 14 days' incubation and sometimes after 35 days, sometimes with heat treatment and sometimes without, for unknown samples, four enrichment samples will be collected on each occasion: samples at 14 and 35 days from tubes that have or have not been subjected to the heat treatment. The experimental work reported above was done by extracting DNA from samples of just 30 µL of enrichment and in most cases these four different samples all yielded essentially the same result. To reduce the amount of work involved in carrying out PCR on these four replicate samples and increase the sensitivity of the assay, for unknown sediment samples, 100 µL of each enrichment sample will be pooled and DNA extracted from the pooled 400 µL. Tests have confirmed that this amount of DNA will not overload the PCR assay.

The inability of the multiplex PCR to detect low levels of spores seeded into sediment (Table 14) adds to the results described in Section 7.2.2 and shows that, at least in our hands, single primers are better at detecting low levels of BoNT-producing organisms. Therefore, once DNA is extracted from an enriched sample, we propose to carry out separate amplifications of each toxin type using the primers of Lindström et al. (2001).

9 Effect of frozen storage

9.1 Background

Freezing has been reported to eliminate some non-botulinal substances for the mouse bioassay (Bott et al. 1968). However, freezing is also claimed to destroy Type E *C. botulinum* (Nickerson et al. 1967). The sediment samples that will be tested in this study were stored chilled (c.a. 4°C) after collection and frozen (-85°C) within a week. They have then been stored at -85°C for up to 6 years. Before conclusions can be drawn about the presence of BoNT-producing spores from these samples, it is important to consider the effect of this freezing step and the frozen storage on their viability.

9.2 Method

1. A stock (16 mL) of each strain was prepared by diluting the original spore crop in saline (0.85% NaCl) such that a 1.0 mL aliquot contained just more than 10^5 spores.

2. Each strain stock was well mixed then dispensed into three tubes (lots) so that each tube contained 4 mL.
3. The first lot of each strain was used to perform serial dilutions of a 4-tube MPN (4x180 µL) using TPYG broth.
4. MPN 96-well plates were incubated at 30°C for 5 days then read by the Multiskan Ex (Thermo Electronic Co-operation) plate Reader.
5. The other two lots of each strain stock were frozen at -85°C overnight.
6. One lot was thawed the next day and MPN (4-tube) calculated to determine the effect of freezing and thawing.
7. The other stock of each strain was transferred to a -20°C freezer and stored for 69 days to determine the effects of accelerated frozen storage (-20 cf -85°C) on recovery.
8. Results for which standard errors did not overlap were considered to be significantly different from each other.

9.3 Results

The effect of freezing and thawing on the recovered spores are shown in Table 13 and Figure 13. Freezing and thawing did not significantly reduce recovery for any of the strains except for Type B17 where recovery was reduced by an MPN of 0.8 $\log_{10}$. For Type A, recovery was slightly improved by an MPN of 0.6 $\log_{10}$ while the effects on the other strains were not significant. After 69 days' storage results were similar (Figure 14).

Table 13: Counts of BoNT-producing strains before and after overnight freezing (-85°C).

Strain	Log MPN before freezing	SE Log MPN before freezing	Log MPN after freezing	SE Log MPN after freezing	Log dif.
A	4.54	0.28	5.12	0.24	0.59
B17	5.33	0.27	4.54	0.28	-0.79
C. but-E	4.30	0.25	4.80	0.25	0.50
E-beluga	4.11	0.24	4.30	0.25	0.19
F	5.12	0.24	5.33	0.27	0.21
F202	5.59	0.05	5.59	0.05	0.00

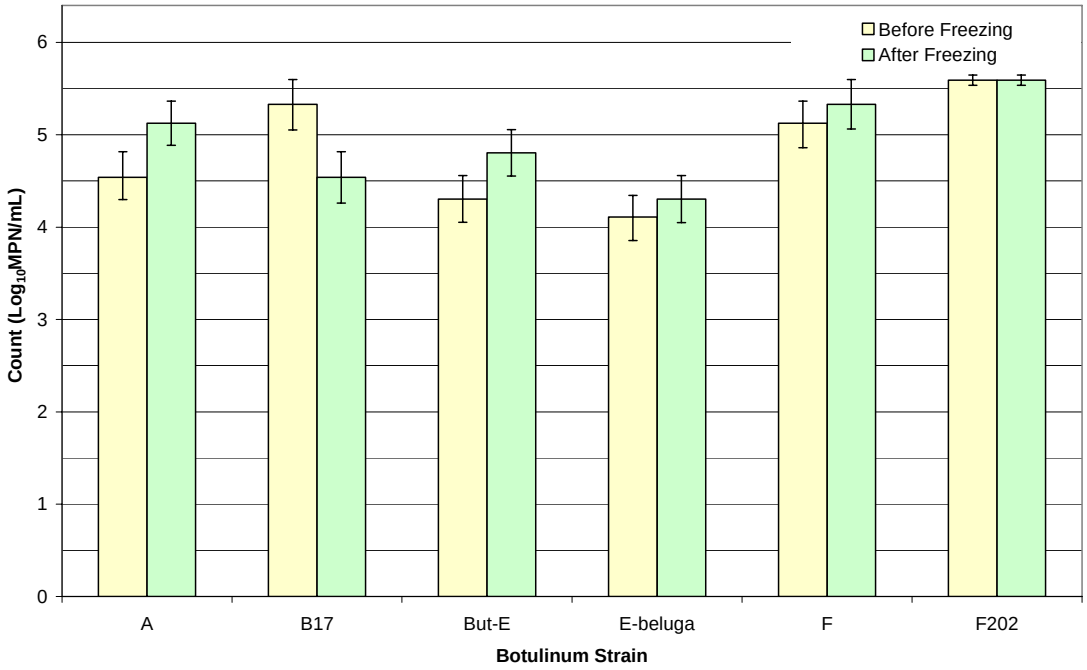

Figure 13: The effect of freezing (-85°C overnight) and thawing on different BoNT-producing spores. Error bars represent standard errors of the log_{10} MPNs.

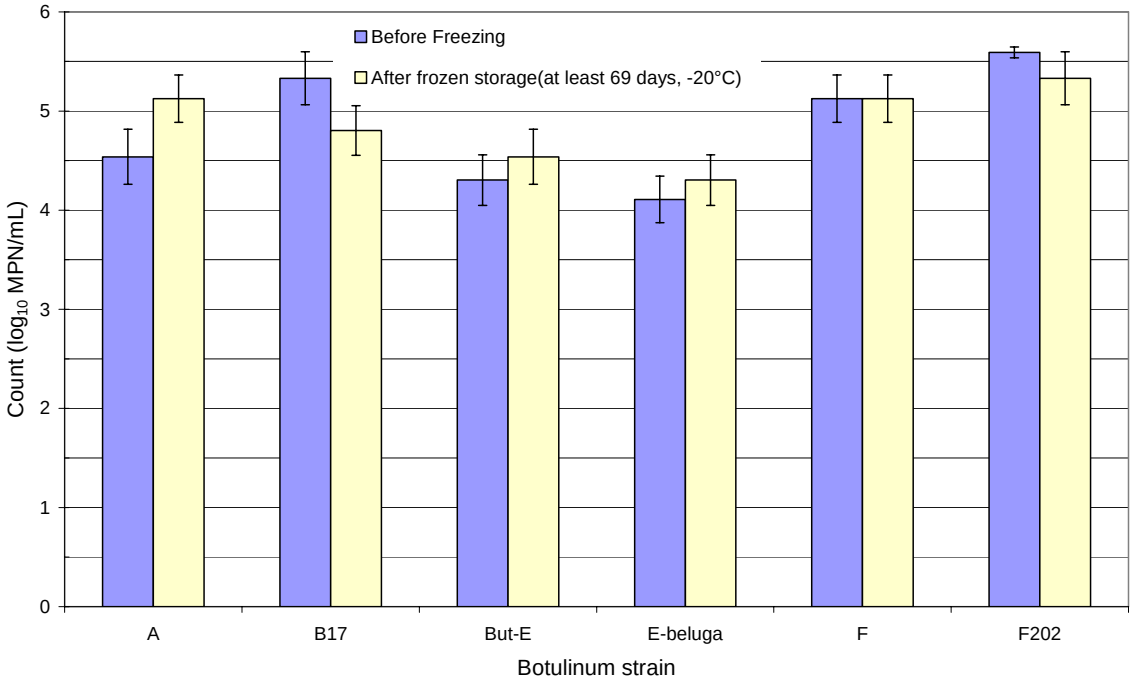

Figure 14. The effect of frozen storage (69 days at -20°C) on different BoNT-producing spores. Error bars represent standard errors of the log_{10} MPNs.

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9.4 Conclusion from freeze-thaw experiment

Freezing and thawing did not affect the viability of spores. The only strain that was negatively affected (B17) was the one that had the lowest detection limit (Table 15) and, even with reduced ability to recover frozen spores, the overall recovery of this strain is likely to be better than that of the other strains. Type A was the strain with the highest detection limit and putting the cells through a freeze-thaw cycle enhanced recovery (probably by enhancing germination).

The results of storage for 2 months at -20°C allow the effect of 6 years' storage at -85°C to be considered. The Arrhenius relationship applies to degradation under frozen storage with a Q_{10} (drop in rate of change in response to a 10°C drop in temperature) ranging from 2 to 20 for most biological systems (Fu & Labuza 1997). Using the least of these ($Q_{10} = 2$), the rate of degradation at -30°C will be 64 times that at -80°C so, at best, 69 days' storage at -20°C equates to more than 12 years at -80°C and more at -85°C.

10 Nested PCR for BoNT type A

10.1 Non-specific amplification

Subsequent to selecting enrichment and detection methods based on the above research, these methods (steps 1 to 3 in section 12 below) were applied to 501 sediment samples and, when amplified with the primers for type A, 247 of them gave PCR bands with similar sizes to those of the positive control for BoNT type A, but when two such bands were sequenced they did not match BoNT type A.

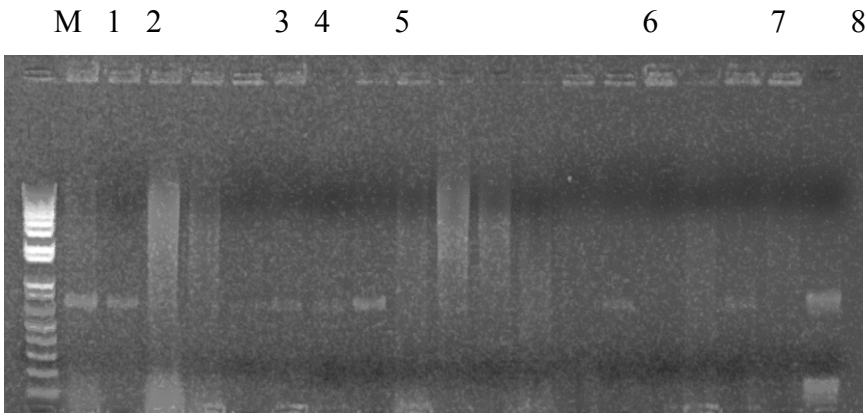

Figure 15: PCR detection of BoNT in some sediment samples after enriching and amplifying with primers for BoNT type A. M = 1 kb plus DNA ladder (Invitrogen); channel 1-7 = probable non-specific amplification of PCR for BoNT type A.; channel 8 = positive control sample for type A (782 bp).

For example in Figure 15 channel 8 shows the control band from a sample spiked with the target BoNT type A gene. The size of the sequence producing this band is 782 base pairs (bp). Channels 1 to 7 from unspiked sediment samples also show bands in this region. Sequencing PCR products from two bands such as these found them to be slightly smaller than expected for type A (759 compared with 782 base pairs) and the closest sequence match was to *Clostridium tetani* rather than *C. botulinum* with an identity match of 80% over 90% of the sequence. Thus, for at least these two samples, the PCR analysis had non-specific amplification resulting in a false positive result. By close examination of the bands in Figure 15 it can be seen that the leading edges of the sample bands are a little lower than that of the control but this is not very convincing. In some cases (e.g. channels 1 and 5) the sizes appear very similar to the control. We therefore chose to develop a nested PCR procedure to confirm positive bands obtained using the type A primer of Lindström et al. 2001.

10.2 Selection of nested primers for BoNT type A

The DNA sequence between the location of 1788 – 2569nt of BoNT/A gene of the reference strain ATCC25763 (accession number EF028391) was retrieved from the Genbank and blasted against the Genbank to search for all the sequences for BoNT/A gene. The alignment of all sequences was carried out using BioEdit software. Two short sequences shared by all the analysed BoNT/A genes were selected as nested primers, as follows:

Forward nested primer: 5'-GGCTGGGTAGAACAATTAG-3'

Reverse nested primer: 5'-AGACCTCATCCCATTTTTC-3'

The primer pair produce a PCR product of 300 bp.

10.3 Nested and semi-nested PCR method

Nested PCR amplifications using the forward and reverse nested PCR primers and semi-nested PCR amplifications using the forward nested PCR primer and reverse PCR primer (CBMLA2) were carried out using PCR products generated by PCR primers CBMLA1 and CBMLA2 as templates. The PCR assays were carried out in a total volume of 20 µl containing 1 µl of template, 0.3 µM concentrations of each primer, 2 µL of 10 x PCR buffer, 2.5 mM $MgCl_2$, 220 µM concentrations of each deoxynucleotide triphosphate, 0.5 U of DNA polymerase (Invitrogen, Carlsbad, USA). The PCR temperature profiles were: 1 cycle of 95°C for 3 min; 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 3 min. The amplified PCR products (10 µl) were visualised in 1% agarose gels by electrophoresis.

10.4 Nested PCR results

Forty seven of the 247 samples gave bands of similar but not quite the same size to those of the BoNT type A control (e.g. Figure 16, lanes 1 to 4 and 9 to 12) and only 1 gave a band with a very close size and intensity match (lanes 4 and 13 in Figure 16).

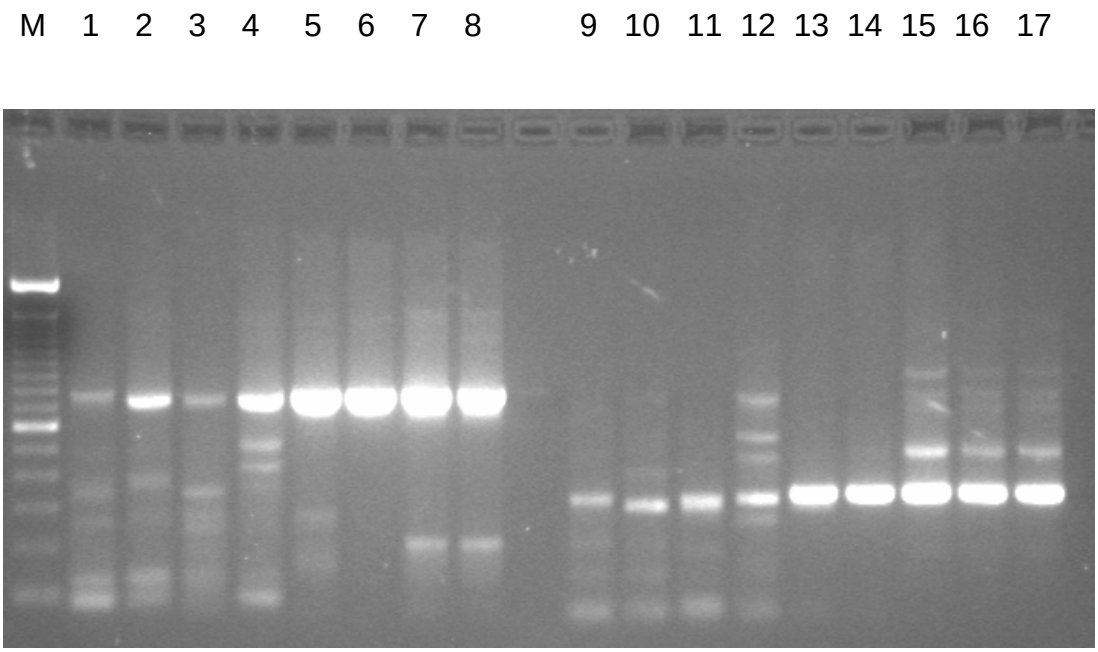

Figure 16. Semi-nested and nested PCR for BoNT Type A. Lanes 1 to 8: semi nested PCR for BoNT type A; lanes 9 to 17: fully nested PCR for BoNT type A. Lanes 5 to 7 and 13 to 17: sediment samples spiked with C. botulinum *Type A, lanes 1 to 3 and 9 to 11: non-specific amplification; lanes 4 and 13: amplification of sediment naturally containing a DNA fragment whose sequence matched BoNT type A.*

When the PCR product from the bands similar to but not the same as BoNT type A was matched with those in the genebank some were best matched to the peptide deformylase gene of *C. botulinum* Types A or F with up to 77% identity match over 100% of the sequenced DNA. However, the PCR product from the sample with a very similar size and intensity searching the genebank database showed that the sequence was that of *C. botulinum* BoNT type A with 91% match to over 100% of the sequenced DNA. There was no significant difference in the specificity of the nested and semi-nested PCR procedures for BoNT type A. However, as subsequent sequencing will be required to confirm the presence of BoNT, the semi-nested PCR will be preferred as a longer DNA fragment will be obtained for sequencing and matching the genebank database.

Thus, the best method to determine whether BoNT type A is present in a sediment sample is to carry out the semi-nested PCR and then sequencing DNA matching the semi-nested PCR of BoNT.

11 Final recommended method

The following method is proposed as a robust method to determine whether spores containing BoNT genes are present in New Zealand marine sediments.

A: Enrichment

1. Sediments stored in 25 mL universal tubes are thawed from • 85°C to room temperature.
2. A 10 g sub-sample is placed in a 125 mL container.
3. Another 10 g is heated (60°C, 10 min) in a 125 mL container and rapidly cooled to 30°C.
4. 90 mL of TPYG is gently added to each 10 g sub-sample followed by gentle mixing and the containers (with loose lids) are sealed in an anaerobic jar using four anerobic generators (to give anaerobic conditions within 2 h).
5. Anaerobic incubation will be for 14 and 35 (assuming the results from the 35-day enrichments are confirmed) days at 30°C.
6. The sediment cultures are thoroughly mixed by inverting. Samples (4 in total) of 100 µL are taken from the two subcultures at day 14 and 35, respectively. The samples taken at day 14 are stored at -85°C until tested after day 35.

B: DNA extraction. Samples taken at both dates are pooled and subject to DNA extraction by FastPrep® bead-beating as follows:

1. The pooled samples are centrifuged (8000 x g, 5 min) and the supernatant removed. The pellet is resuspended in 0.1 M phosphate buffer (200 µL, pH 7.4) and mixed by vortexing. The pooled samples are centrifuged again (8000 x g, 5 min) and the supernatant removed.
2. The pellet is resuspended in 0.1 M phosphate buffer (270 µL, pH 7.4) with added lysozyme (30 µL, 50 mg/mL). This suspension is mixed by vortexing and incubated (37°C, 30 min) followed by the addition of Lysis buffer (300 µL, 0.1M NaCl, 0.5M Tris, pH 8.0, 10% SDS) and 300 µL chloroform: isoamyl alcohol (24:1). The suspension is then mixed and transferred into a 2-mL Screw-top Micro tube (Cat. #72693005, Sarstedt, Germany) containing 100 mg of 0.1 mm Zirconia/silica beads (Catalog #110791012, Biospec products Inc.) and 100 mg of 2.5 mm Zirconia/silica beads (Catalog #110791252, Biospec products Inc.). The mixture is run on the FastPrep® FP220A Instrument (Qbiogene, CA, USA) at 4.5 m/s for 40 s.
3. The supernatant (about 700 µL) is transferred to an Eppendorf tube followed by addition of 7 M NH4Ac (390 µL). The suspension is mixed by vortexing and centrifuged at 13 000 x g for 8 min. The supernatant (about 830 µL) is transferred to another Eppendorf tube, and isopropanol

(448 µL) is added and thoroughly mixed by repeatedly inverting the tube. The mixture is left to stand (room temperature, 1 min) and then centrifuged (3000 x g, 10 min). The supernatant is decanted and the pellet is washed with ice-cold ethanol (750 µL), centrifuged (13 000 x g, 10 min) and the DNA in the pellet air-dried and resuspended in 20 µL H_2O). This is stored (4 or -20°C) for PCR use.

C: PCR amplification

1. Two µL from each DNA sample are used for separate PCR amplification for each of the four BoNT genes (A, B E, and F) using the primer sets described by Lindström et al. (2001).
2. The PCR mix contains the following reagents: 2 µL of DNA template, 2 µL of 10 x PCR buffer, 0.3 µM of each primer, 2.5 mM MgCl2, 220 µM concentrations of each deoxynucleotide triphosphate, 0.5 U of DNA polymerase (Invitrogen, carlsbad, USA) in a total volume of 20 µL.
3. The PCR is carried out using the following temperature profiles: 1 cycle of 95°C for 3 min; 35 cycles of 95°C for 30 s, 60°C for 25 s, and 72°C for 60 s; and 1 cycle of 72°C for 3 min.
4. The amplified PCR product (5 µL) is loaded on to 1% agarose gels for electrophoresis analysis for the presence of the BoNT genes.

D: Nested PCR. If bands matching type A are detected, these will be subjected to nested and semi-nested PCR:

1. The bands are excised from the gels and subjected to nested PCR base using the following primers:
   - Forward primer: 5'-GGCTGGGTAGAACAATTAG-3'
   - Reverse primer: 5'-AGACCTCATCCCATTTTTC-3'

   And to semi-nested PCR using the forward primer above and the reverse PCR primer (CBMLA2) of Lindström et al. (2001).
2. The PCR reaction mixture contained 1 µL PCR product is mixed with 2 µL of 10 x PCR buffer, 2.5 mM $MgCl_2$, 220 µM concentrations of each deoxynucleotide triphosphate, 0.5 U of DNA polymerase (Invitrogen, Carlsbad, USA), 0.3 µM of each nested primer for fully nested and just the forward primer for semi-nested PCR in a total volume of 20 µL.
3. The PCR is carried out using the following temperature profiles: 1 cycle of 95°C for 3 min; 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 3 min.
4. The PCR products (10 µL) are then loaded on to 1% agarose gels for electrophoresis analysis for the presence of the BoNT genes.

Where the PCR of types B, E and F or nested and semi-nested PCR of type A gives bands of a molecular weight similar to those from controls containing the respective BoNT type A they are subjected to sequencing.

1. The band is excised from the gel and DNA purified using Wizard® SV Gel and PCR clean-up system (Promega, Madison, USA).

2. The purified PCR products are sequenced by the Allan Wilson Centre, Massey University, Albany.
3. The obtained DNA sequence is blasted against Genbank to search for its homologue and to compare its similarity with sequences lodged in the Genbank.

This method should detect any BoNT-producing spores present in the samples down to a level of at most 15 spores per g but probably lower. It will not be so effective at detecting vegetative cells.

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