PRESENCE AND PERSISTENCE OF THE HAZARDOUS MICRO-ORGANISM *C.botulinum* IN BALLAST SEDIMENTS

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Summary

Ballast water from ships has been shown to be responsible for the transfer of marine organisms including toxic dinoflagellates into harbours previously free from those organisms. The possibility therefore exists that *Clostridium botulinum* (the causative organism of the potentially fatal disease, botulism) could be introduced in a similar way. There are seven types of *C.botulinum* (designated A - G) and types A, B and E most commonly affect man. Information regarding the distribution of *C.botulinum* in Australian soils and marine sediments is limited but *C.botulinum* type E has so far, not been found. The possible introduction of *C.botulinum* type E into our waters could pose a considerable threat to our fishing industry since this organism is capable of growth and toxin production at temperatures as low as 3.3°C (i.e. refrigeration temperatures).

Many of the ships entering Australian ports take on ballast from areas where *C.botulinum*, particularly type E, is endemic. This study was undertaken to determine the incidence of *C.botulinum* in the ballast of ships entering Australian ports and to study harbour sediments for the presence of *C.botulinum.* Studies were also made of the ability of *C.botulinum* to survive in ballast samples inoculated with spores of *C.botulinum* and stored at temperatures comparable with those encountered during a sea voyage.

One of the 281 ballast samples assayed contained *C.botulinum* type C. That sample was taken from a ship which originated in Haldia, Norway, then docked in Singapore, Malaysia before arriving in Gladstone, Queensland. The ballast tanks were sampled by the Australian Quarantine Inspection Service. Fifty seven harbour samples were also examined: none of those contained *C.botulinum.*

BACKGROUND

The spore forming bacterium *Clostridium botulinum* produces botulinum toxin, the toxin responsible for the potentially fatal disease botulism. Victims of non-fatal cases of botulism suffer prolonged and debilitating neurological symptoms. This microorganism is common in soils and sediments in some parts of the world and is of major public health significance when present in food. Many food processing and storage procedures are designed specifically to destroy or inhibit *C.botulinum*. It is of particular concern to fisheries industries overseas because some types (psychrotrophic types) found in aquatic environments can grow at temperatures as low as 3.3°C. Chilled fishery products are likely to be held at temperatures above 3.3°C on occasions, leading to a risk that *C.botulinum* will grow and produce botulinum toxin. Many fatal cases of botulism have been caused by fishery products. Several outbreaks of botulism caused by fishery products are reported per year in Japan.

There have been no recent surveys of Australian soils or marine environments for the presence of *C.botulinum* and it is possible that changed agricultural practices or new manufacturing and processing procedures may have introduced, or favoured the development of *C.botulinum* in some environments.

There is considerable evidence that a variety of marine organisms have been introduced into Australian waters via ballast sediments brought from other countries in ships. Ballast water is taken on by vessels in their port of origin and this ballast may contain sediments. The ballast (including the sediment) is then discharged by those vessels on arrival in Australian ports. Prior to the late 1980's dinoflagellates known to produce paralytic shellfish poisoning had not been reported in Australian waters. Between 1986 and 1987 three species of toxic dinoflagellate were found for the first time in Australian estuarine waters - around Tasmania, Adelaide and Melbourne (Hallegraeff, Steffensen and Wetherbee, 1988). A preliminary survey, indicated that ballast sediments from three of six ships arriving at Triabunna contained viable dinoflagellate spores (Hallegraeff, Bolch, Koerbin and Bryan, 1988) and cysts of the

toxic dinoflagellate *Alexandrium* cf. *tamarense* were found in ballast sediment from Japanese tankers (Hallegraeff, Bolch, Bryan and Koerbin, 1990; Hallegraeff and Bolch, 1991).

Some of the vessels entering Australian ports take on ballast in areas of the world where *C.botulinum* is endemic and there is no reason to believe that *C.botulinum* could not also survive in those ballast sediments. Introduction of this organism to our marine environment would have serious safety implications for Australian seafoods and handling and processing procedures would need to be modified.

The strains of *C.botulinum* are classified into 7 types (A - G), depending on the serological specificity of their neurotoxin. Types A, B and E most commonly affect man, although all types of *C.botulinum* are capable of affecting man. The natural reservoir for this organism is the soil and it therefore finds its way onto raw agricultural products such as fruit, vegetables, meat and fish.

Most of the reported soil surveys were conducted in North America, USSR and Europe. Relatively large numbers of spores were found in soils sampled in the vicinity of aquatic environments (i.e. along inland rivers and lake shores and near fish ponds. Type E spores predominated although types A, B, C, D and F were also found. Type E *C.botulinum* spores were also the prevalent type found in the interior of Sweden and Hokkaido, Japan (Hauschild, 1989).

There is comparatively little information about *C.botulinum* in soils from Australia and psychrotrophic types of *C.botulinum* are thought to be rare or absent in Australian marine environments. A survey of 528 samples of mud, soil, potato washings and fish intestines from NSW, Tasmania and Queensland yielded no *C.botulinum* type E (Christian, 1971). *Clostridium botulinum* types A, B, C and D have occasionally been found in Australian soils (Eyles and Warth, 1981).

Clostridium botulinum type E is frequently isolated from the marine environment, particularly in the cooler waters of the east and west coast of North America and the North Sea and Baltic Sea coasts of Europe. Type E *C.botulinum* has also been isolated from coastal sediments of Iran, Japan, Thailand and Indonesia. *Clostridium botulinum* (types A-E and occasionally F) were also isolated from fish and invertibrates caught in the same waters. Again *C.botulinum* type E was most frequently isolated (summarized from Hauschild, 1989).

The non-proteolytic types of *C.botulinum* comprise type E and some strains of type B and F, they are also psychrotolerant and capable of growth and toxin production at temperatures as low as 3.3°C (Eklund, Wieler and Poysky, 1967; Eklund, Poysky and Wieler, 1967; Schmidt, Lechowich and Folinazzo, 1961). The potential for botulism from raw fish is therefore great, particularly if shelf-life is increased by e.g.. vacuum packaging or modified atmosphere storage as these types of *C.botulinum* are capable of growth and toxin production even at normal refrigeration temperatures. Further processing such as smoking, salting etc. does not necessarily destroy the spores and subsequent bad storage conditions could result in their outgrowth and toxin production (Eklund, 1982).

Clostridium botulinum has been isolated from commercially prepared and processed fish. Frozen, vacuum packed flounder and white fish chubs from the USA, and vacuum packed fish from the UK contained Type E. Marine fish from Japan contained *C.botulinum* type C and in one instance type D. Salted fish from the Caspian sea and smoked fish from the Pacific coast of the USA and the Caspian Sea and smoked Eel from the Baltic sea also contained type E. Smoked salmon from Denmark contained *C.botulinum* type B and smoked white fish chubs from the Great Lakes contained types E and B (summarized by Hauschild, 1989).

This project sets out to determine the incidence of *C.botulinum* in ballast sediments and our coastal marine environment. Also to study the survival of inoculated spores of *C.botulinum* in ballast sediments at a range of temperatures comparable with those encountered in the ships ballast tanks during a sea voyage. The results gained will provide the fishing industry with

relevant information to ensure the continued safety of our fish and fishery products.

METHODOLOGY

Samples

Samples were obtained from the ballast tanks of ships entering Australian ports between 11/12/87 and 30/3/90. The samples were initially obtained by the Australian Quarantine and Inspection Service (AQIS) for examination for the presence of toxic dinoflagellates and were kindly supplied by Dr G.Hallegraeff (CSIRO Division of Fisheries Research, Marine Laboratories, GPO Box 1538, Hobart, Tasmania 7001). Details of the samples are given in Table 1. Harbour samples, obtained from sediments in Australian ports as well as control samples from other estuaries not exposed to commercial shipping were also collected. These samples were also provided by Dr G Hallegraeff, and were initially obtained by AQIS. They are listed in Table 2.

Media

Details of the media used are described in Appendix I.

Detection of vegetative cells or spores of *C. botulinum*

Approximately 10g of each ballast or sediment sample was inoculated into freshly prepared TPYGC broth then incubated at 30°C for 48 - 72h. The culture filtrate was centrifuged and 0.5 ml aliquots injected into mice which were observed over a period of 4d for symptoms typical of *C.botulinum* intoxication. Culture filtrates causing death of one or both replicate mice were investigated further to confirm the presence of *C.botulinum* toxin. Presence of the toxin indicates growth of *C.botulinum* and therefore its presence in the original sample. Once the presence of *C.botulinum* toxin was confirmed attempts were made to isolate the organism. Further details of toxin testing and isolation methods are provided in Appendix I.

Evaluation of trypticase medium for the isolation of *C.botulinum* from ballast

Ballast samples 414, 429, 439, 442, 470 and 475 were used for these experiments. Several ballast samples were necessary as there was insufficient ballast from any one sample for the whole experiment. Approximately 10g of ballast was inoculated into sterile (28ml) bottles. Each bottle was then inoculated with 1ml of culture filtrate that had been previously heated to destroy any toxin or vegetative cells. The following strains were used as the inoculum: *C.botulinum* type A (strain 121, proteolytic), type B (strain 7273B, proteolytic and strain 17B, non-proteolytic), type E (strain Minneapolis) and type F (strain 202, non-proteolytic). All samples were stored at 4°C for 7 days then inoculated into duplicate trypticase broths, one replicate of which contained 0.1% trypsin, all broths were incubated at 30°C/3d then tested for the presence of *C.botulinum* toxin.

Survival of inoculated spores *C.botulinum* during storage of ballast

Nine ballast samples containing sediment were further studied to estimate the total numbers of anaerobic bacteria present. Samples containing the lowest anaerobic counts were then used in the inoculation experiments. Three media were used to enumerate the anaerobic bacteria - RCA to give a total anaerobic count, RCA + cycloserine, and RCA + egg yolk. *Clostridium botulinum* is resistant to cycloserine and it was hoped that the antibiotic would inhibit some of the other anaerobic organisms present in the ballast sample thus permitting selective enumeration of *C.botulinum*. *C.botulinum* also produces typical reactions on agar containing egg yolk and this was added to attempt differential isolation of *C.botulinum* in the presence of other organisms.

The following strains of psychrotolerant, nonproteolytic *C.botulinum* were used for inoculation of the ballast sediments: Type B, strain 17B, type E, 'Minneapolis' and type F, 202F. Sporulation was attempted in two media, TPYGC broth incubated at 30°C and RCA incubated anaerobically at 30°C, broths and plates were examined every 2-3 days for the

presence of spores. Full details of the strains used and method of production are given in Appendix I.

When sufficient spores had been obtained the suspensions were counted to estimate the number of spores present. Decimal dilutions were prepared using 0.1% peptone water and dilutions plated onto RCA using the pour plated method. Plates were incubated anaerobically at 30°C/2d and colonies counted.

For each inoculation experiment approximately 10g of ballast sample was placed into each of five sterile (28ml) bottles. Four bottles were then inoculated with 1ml of spore crop. 1ml of sterile water was added to the fifth bottle which served as the uninoculated control. The samples were well mixed and 1ml removed immediately from each bottle for the time zero count. The four inoculated ballast samples were then incubated at either 5, 10, 15 or 20°C for up to a month. The uninoculated sample for the type B experiment was stored at 20°C and that for the type E experiment was stored at 5°C. Counts were made at approximately 1 week intervals during storage.

RESULTS

Evaluation of trypticase medium for the isolation of *C.botulinum* from ballast

Results of the toxin tests from trypsinised and untrypsinised enrichment broths inoculated with culture supernatents of known strains of *C.botulinum* are shown in Table 3. For all strains A - E toxin was detected and confirmed by neutralization tests from both trypsinised and untrypsinised enrichments, indicating that the medium chosen was capable of isolating *C.botulinum* and that the incorporation of trypsin into the medium was not necessary. For strain 202F, toxin was detected in low levels from both trypsinized and untrypsinized medium, but subsequent neutralization tests showed toxicity of the sample had been lost. There were insufficient mice available for this test to be repeated but the preliminary tests indicated that toxin was produced in both trypsinized and untrypsinized medium.

Survival of inoculated spores *C.botulinum* during storage of ballast

Total anaerobic counts from the 9 ballast samples studied are presented in Table 4. None of the three media tested was consistently more selective, although occasional differences between the counts were noticed. The simplest medium (RCA) was therefore chosen as the isolation medium for the inoculation experiments. Ballast samples 439, and 470 showed the lowest anaerobic counts on all three media.

The following spore crops were prepared: *C.botulinum* type B (non-proteolytic) 5.8 x 10^7 spores/ml and *C.botulinum* type E 9.1 x 10^5 spores/ml. After many unsuccessful attempts preparation of spore crops of *C.botulinum* type F were abandoned.

The results of the ballast inoculation and storage tests are presented in Table 5 (*C.botulinum* type B) and Table 6 (*C.botulinum* type E).

C.botulinum type B inoculum

A mean count of 5.5 x 10^5 was obtained for the four replicate samples which was almost 2 log cycles higher than the background count of the uninoculated ballast (8.6 x 10^3). Throughout the 28 day storage period there was no significant change in numbers for either the uninoculated or inoculated ballast samples indicating that *C.botulinum* type B did not grow in the ballast sample during storage irrespective of temperature. Additionally there was no decrease in numbers indicating that the spores did not die during storage in ballast at temperatures between 5 and 20°C over a 28 day period. Since no heat treatment was given to these samples the presumptive *C.botulinum* count comprises either spore or vegetative cells or a mixture of the two. In order to establish whether the spores of *C.botulinum* germinated during that time an additional heated count was also made to estimate the numbers of spores present. Again numbers of spores did not change significantly during the storage period and were not significantly different from the unheated count indicating that the inoculated spores of *C.botulinum* type B remained unchanged in the ballast throughout the experiment. Confirmatory tests on the colonies counted as presumptive *C.botulinum* were not carried out since the counts from the inoculated samples remained in the order of 5 x 10^5 (two log cycles higher than the uninoculated count. The higher count was therefore attributed to the inoculated *C.botulinum*.

C.botulinum type E inoculum

The background count of the ballast sample for this experiment was about 3 x 10^4 which was one log higher than for the type B experiment. Additionally the count after inoculating with type E spores was not significantly higher for the time zero unheated count. Once storage had begun the inoculated samples showed counts approximately one log higher than the uninoculated for the day 10 and 19 samples and for some day 26 samples. Considerable variation in rates of germination of spores of *C.botulinum* is well documented and it is possible

that in this experiment some of the spores inoculated were unable to germinate thus giving lower counts from some samples than others. The mild heat treatment (given to destroy vegetative cells) may have been sufficient to stimulate germination of some of those inoculated spores, hence higher counts were seen after heating. Since there was no change in the numbers from the uninoculated ballast samples (either heated or unheated) it can be assumed that the counts of 1 x 10^5 relate to the inoculated *C.botulinum* type E.

The heated inoculated count was approximately 1 log higher (2 x 10^5) than the heated uninoculated count. Numbers for the heated inoculated ballast samples remained similar throughout the storage period, indicating that between 5 and 20°C storage there was no change in numbers of *C.botulinum* type E spores over the 33 day storage period.

Ideally higher numbers of inoculated spores and a lower background count of ballast is necessary to be sure of detecting changes in the level of inoculated *C.botulinum* spores. Considerable difficulty was encountered in obtaining sufficient numbers of spores. Time restrictions dictated the experiment proceed even though a higher number of spores would have been preferable.

Incidence of *C.botulinum* in Ballast Samples

A total of 281 ballast samples were tested for the presence of botulinum toxin. Enrichment cultures from four of those samples (code 416, 444, 461 and 472) initially caused death in mice. The results of those and further confirmatory tests are presented in table 3.

Sample 461 caused death of one mouse after approximately 40min, such rapid death is unlikely to be caused by *C.botulinum* toxin therefore the test was repeated using duplicate mice. None of those mice died and no further work was carried out on that sample.

Samples 416 and 444 caused death of a mouse after 21 and 45h respectively. Those samples were injected into duplicate mice after diluting with gelatin phosphate buffer and polyvalent *C.botulinum* antitoxin. None of the mice receiving those diluted samples died. Since the deaths were non-specific no further investigations were made.

Sample 472 caused death in one mouse within 20h, duplicate mice injected with similar samples also died within approximately 22h. The sample was then diluted with either buffer or polyvalent *C.botulinum* antiserum and injected into duplicate mice. Mice receiving sample plus buffer died whereas those receiving sample plus polyvalent antiserum were protected. Neutralization tests were then carried out using specific antiserum to the different types of *C.botulinum.* Those tests confirmed the presence of *C.botulinum* type C.

Ballast sample 472 came from the starboard top tank 2 of the vessel "Vivita" which departed Haldia, Norway on the 27/2/90, the ship docked in Singapore, Malaysia, and departed on 17/3/90. It arrived in Gladstone, Queensland, Australia 20/3/90 and the tanks were sampled 21/3/90.

Incidence of *C.botulinum* in Harbour Samples

Samples C and E36 initially caused death in one mouse. The results of those and further confirmatory tests are presented in Table 7.

For the mouse receiving sample C, mild symptoms typical of *C.botulinum* intoxication were noted after 23h and the mouse died after 31h. Sample C was diluted with buffer or *C.botulinum* antiserum to types A, B and E then injected into further mice. All of those mice survived and the death of the initial mouse was therefore assumed to be non-specific.

Sample E36 caused the death of a mouse within 20h, a further sample was then injected into duplicate mice. Those mice survived without showing symptoms of *C.botulinum* and the initial death was again assumed to be non-specific.

None of the 57 harbour samples tested, were found to contain *C.botulinum.*

SIGNIFICANCE OF *C.botulinum* TYPE C IN BALLAST

Generally it is assumed that *C.botulinum* type C only affects animals. It is a common source of botulism in wildfowl, broiler chickens and cattle. It has also affected a wide range of animals, including dogs, that may consume contaminated carcasses found in the wild. However there have been several cases of suspected human type C botulism: In the 1950's two cases were reported; in the USA (Meyer *et al.*, 1953) and France (Prevot *et al.*, 1955). In both outbreaks type C *C.botulinum* was isolated from incriminated food, and symptoms typical of botulism were apparent in those affected but no serological evidence was provided. In 1967, Matveev *et al.* reported two cases of human type C botulism in Russia between 1964 and 1966, but offered no details. In 1983, two of the several cases of botulism reported in Belgium, appeared to be caused by both *C.botulinum* types B and C. Few details were given, but serum from both cases contained a mixture of types B and C toxin. Type B toxin only was found in the incriminated food (Marchal, et al., 1985).

Although none of the above cases were proven *C.botulinum* type C botulism, it appears likely that *C.botulinum* type C is capable of occasionally affecting man.

There is no evidence to suggest that type C *C.botulinum* is more resistant to physical stress, such as heating and resistance to preservatives (sodium chloride, nitrite) than other types of *C.botulinum* (Roberts and Gibson, 1979). However its toxin may be more resistant to dilution in water (Brygoo, 1953; Prevot and Brygoo, 1953; Haagsma, 1973; Graham *et al.*, 1978) and

more resistant than types A, B and E to heat (Prevot and Brygoo, 1953). Roberts and Gibson (1979) concluded that type C *C.botulinum* posed no additional threat to the food industry and measures to inactivate or control the other types of *C.botulinum* should also control type C.

In the natural environment type C has been isolated from fish and invertibrates from the following waters: East coast of the USA from New York to Florida, the Gulf coast, Caribbean, Gulf of Venezuela and the Brazilian coast south of latitude 24°S and approximately half of the fish and invertibrates sampled from waters around West Indonesia, Java and the Gulf of Siam contained type C *C.botulinum*. The minimum growth temperature for type C *C.botulinum* was reported as 12.8°C for terrestrial strains and 15.6°C for marine strains (Segner, Schmidt and Boltz, 1971). Thus good refrigeration should prevent its outgrowth, however the potential for growth, should refrigeration conditions fail remains similar to that for other types of *C.botulinum*.

Since the minimum growth temperature for *C.botulinum* type C varies between 12.8 - 15.6°C it is unlikely that growth of this strain occurred in the ballast during the voyage. However the fact that this organism was isolated from the ballast, together with the fact that spores of *C.botulinum* type B inoculated into ballast survived for at least 28 days at temperatures between 5 and 20°C indicates that spores of *C.botulinum* are capable of surviving in the ballast water during long sea voyages and that concerns that other types of *C.botulinum* such as type E may be transported in a similar way are justified.

It was expected that a higher proportion of ballast samples would have contained *C.botulinum*, since all samples tested contained sediment. The amount and type of sediment varied considerably from fine brown or black mud which quickly settled out to almost solid mud. Occasional samples contained wood chips, iron flakes or rocks.

Studies carried out by Hallegraeff and Bolch (personal communication) using the same ballast samples showed that although both benthic and planktonic organisms were present in

many samples by far the greater proportion of organisms present comprised planktonic diatoms. Material therefore enters the ballast tanks predominantly from the water column and resuspended sediment comprises only a small proportion of the total ballast. If that is the case a low incidence of *C.botulinum* may be anticipated since *C.botulinum* would be expected to be present in the sediment rather than the water column.

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Table 1. Ballast Samples Tested

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port*
2	Meridian	4	Kushiro(Japan)	21/11/87	11/12/87	Spring Bay(Tas.)
3	Forest Trader	-	Yatsushiro(Japan)	19/12/87	6/1/88	Spring Bay
5	Forest Trader	-	Ishinomaki(Japan)	20/2/88		Spring Bay
6	Jujo Maru	-	Kushiro	-	26/2/88	Spring Bay
8	Shearwater	fore d	Yatsushiro	10,13/2,4/3/88	25/3/88	Spring Bay
9	Jujo Maru no.2	-	Kushiro	26/3/88	13/4/88	Spring Bay
13	Forest Trader	-	Yura	-	16/8/88	Spring Bay
14	Kunisaki Maru	stb 5	Kimitsu	7/7/89	1/7/89	Port Hedland
15	Kunisaki Maru	stb 6	Kimitsu	7/7/89	1/7/89	Port Hedland(WA)
16	New Era	top 1	Colombo(Sri Lanka)	19/6/89	1/7/89	Port Hedland
17	Wendeng Hai	4	Hong Kong	6/6/89	27/6/89	Port Hedland
18	Samoa	w 3,4	Ciquading(Indonesia) -		5/3/89	Gove(NT)
19	Nikkei Challenge	stb	Shimizu(Japan)	22/4/89	4/5/89	Gove
20	Massey Phoenix	stb 3	Quinhuangdao	22/2/89	8/3/89	Gove
21	Otaru Rex	-	Jeddah	-	7/5/89	Hobart(Tas.)
22	Otaru Rex	-	Jeddah	-	7/5/89	Hobart
23	Mulberry	-	Kushiro	27/5/89	13/5/89	Spring Bay

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
32	Massey Phoenix	2	Quinhuangdao	22/2/89	8/3/89	Gove
35	Spring Condor	fpt	-	-	6/7/89	Port Hedland
36	Pacific Jasmin	stb w7	Kashima(Japan)	5/6/89	27/6/89	Port Hedland
37	Eb Trader	2	Kaohsiung(Taiwan)	6/6/89	29/6/89	Port Hedland
38	New Era	top 6	Colombo	19/6/89	1/7/89	Port Hedland
41	Kohkisan Maru	-	Kawasaki(Japan)	31/5/89	16/6/89	Port Hedland
42	Kohkisan Maru	-	Kawasaki	31/5/89	16/6/89	Port Hedland
43	Eb Trader	5	Kaohsiung	6/6/89	29/6/89	Port Hedland
44	Pacific Jasmin	pt w7	Kashima	5/6/89	27/6/89	Port Hedland
45	Nichibu Maru	top 4	-	-	-	-
46	Silver Sorrel	top 4	-*	-	-	-
47	Phatanus	fpt	Japan	-	-	-
49	Brisas 1	fpt	-	-	7/8/89	Newcastle(NSW)
50	Brisas 1	fpt	-	-	7/8/89	Newcastle
51	Iron Newcastle	-	-	-	17/7/89	Newcastle
52	Shinei Maru	pt w4	Nagoya(Japan)	15/7/89	1/8/89	Newcastle
53	Shinei Maru	fpt	Nagoya	15/7/89	1/8/89	Newcastle

* Last cleaned and reballasted in Mexico, sampled in Honduras 9/5/89

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
56	Nand Shristi	b 4	Singapore	18/5/89	28/5/89	Bunbury
58	Nand Shristi	b 4	Singapore(Malaysia)	18/5/89	28/5/89	Bunbury
72	Daishowa Maru	-	Muroran(Japan)	-	20/7/89	Eden(NSW)
73	Daishowa Maru	-	Shimizu	-	28/8/89	Eden
75	Shinei Maru	1	Osaka(Japan)	20/6/89	17/7/89	Port Hedland
78	Atsuta Maru	-	Fukuyama(Japan)	19/6/89	6/7/89	Port Hedland
79	Western Nine	pt db 4	Pohang(Sth. Korea)	5/7/89	30/7/89	Port Hedland
80	Western Nine	6	Pohang	5/7/89	30/7/89	Port Hedland
81	Hyundai Continental	4	Pohang	8/7/89	1/8/89	Port Hedland
82	Hyundai Continental	-	Pohang	8/7/89	1/8/89	Port Hedland
86	Kohkisan Maru	stb w4	Fukuyama	7/7/89	18/7/89	Port Hedland
87	China Steel Realist	pt 6	Kaohsiung(Taiwan)	6/7/89	29/7/89	Port Hedland
88	China Steel Realist	pt 6	Kaohsiung	6/7/89	29/7/89	Port Hedland
89	Donau Maru	-	Kisarazu(Japan)	18/6/89	18/7/89	Port Hedland
90	Donau Maru	w 1	Kisarazu	18/6/89	18/7/89	Port Hedland
92	Kohkisan Maru	stb w4	Fukuyama	7/7/89	18/7/89	Port Hedland
93	M.V. Poquita Mami	3	Shibushi(Japan)	25/5/89	14/6/89	Launceston

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
94	Shin Sendai	4	Mutsure(Japan)	9/3/89	2/4/89	Launceston
95	M.V. Neo Honeysuckle	deep 1	Kaohsiung	3/6/89	22/6/89	Launceston
97	M.V. Iran Aol	stb w1	Bandar Abbas(Iran)	-	20/4/89	Geelong
98	M.V. Neopelargonium	w 2	Tampa(USA,Fla.)	-	7/4/89	Geelong, Vic
99	M.V. General Mascardo	pt top4	Vancouver(Canada,BC)	23/3/89	18/4/89	Geelong
100	M.V. Nand Rati	db 3	Los Angeles(USA,Cal.)	1/3/89	29/3/89	Geelong
101	M.V. Iran Aol	pt top1	Bandar Abbas	-	20/4/89	Geelong
103	M.V. Neopelargonium	w 3	Tampa	-	7/4/89	Geelong
104	M.V. Nand Rati	db 3	Los Angeles	1/3/89	29/3/89	Geelong
106	M.V. General Mascardo	pt w5	Vancouver	23/3/89	18/4/89	Geelong
108	Yick Fat	stb w5	Quinhuangdao	26/3/89	10/4/89	Newcastle
109	Forest Prince	4	Sakaiminato(Japan)	-	24/6/89	Launceston
116	Eastern Plum	-	Kaohsiung	17/5/89	2/6/89	Port Latta(Tas.)
117	Eastern Plum	-	Kaohsiung	17/5/89	2/6/89	Port Latta
118	Iron Newcastle	4*	-	-	17/7/89	Newcastle
119	Philippine Sampaguita	fpt	Sodegaura(Japan)	-	12/7/89	Newcastle
121	Growth Ring	4	Tsuneishii(Japan)	-	1/3/89	Spring Bay

* near no 4 hold.

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
125	Meridian*	-	Yatsushiro	-	14/8/89	Spring Bay
126	Meridian*	-	Yatsushiro	-	14/8/89	Spring Bay
129	Craig the Pioneer	deep 4	Iwakuni(Japan)	-	13/7/89	Launceston
131	Pacific Taio	4	Iyomishima(Japan)	-	23/4/89	Launceston
132	Port Kembla Sediment	-	Kembla Harbour(Aust.)	7/8/89	-	-
137	Iron Kembla	stb 5	-	-	7/8/89	Port Kembla(NSW)
138	Fruiton	-	Quinhuangdao	-	12/5/89	-
139	Zirje	w 2	Puerto Acevedo(Arg.)	20/3/89	5/5/89	Geelong
140	Zirje	w 4	Puerto Acevedo	20/3/89	5/5/89	Geelong
141	Meridian	4	Yatsushiro	5/6/90	27/6/89	Geelong
142	Sunvil	w 2	Tokyo	1/6/89	15/6/89	Geelong
144	Gokosan	w 3	Kobe(Japan)	7/6/89	25/6/89	Geelong
145	Gokosan	w 4	Kobe	7/6/89	25/6/89	Geelong
146	Meridian	4	Yatsushiro	5/6/89	27/6/89	Geelong
148	Eiyo Maru	4	Iwakuni	29/6/89	13/7/89	Bunbury
149	Nan Feng	top 1	Singapore	5/7/89	14/7/89	Bunbury
150	Yong Jiang	6	Singapore	8/7/89	18/7/89	Bunbury

* reballasted at sea

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
152	Eiyo Maru	fpt	Iwakuni	29/6/89	13/7/89	Bunbury
153	Nan Feng	top 1	Singapore	5/7/89	14/7/89	Bunbury
154	Yong Jiang	2	Singapore	8/7/89	18/7/89	Bunbury
162	Hokoetsu Ace	4	Niigata(Japan)	15/7/89	3/8/8	Bunbury
164	Carlo M	top 5	-	-	4/8/89	Bunbury
170	Ming Courage	-	Fukuyama	-	20/4/89	Newcastle
171	M.V. Pernas Amancy	-	Osaka	-	19/4/89	Newcastle
172	General Hizon	-	Washington(USA)	-	6/4/89	Newcastle
174	M.V.Thalassini Avra stb 5		Pohang	-	20/4/88	Newcastle
175	Daishowa Maru	-	Shimizu	-	17/3/89	Eden
176	Taio Dream	4	Mishima(Japan)	3/3/89	20/3/89	Launceston
177	Hachinohe Maru	4	Hachinohe(Japan)	2/3/89	22/3/89	Launceston
178	Pacific Taio	4	Iyomishima	23/2/89	14/3/89	Launceston
179	Grace Taio	-	Iyomishima	25/2/89	25/2/89	Launceston
183	Eden Maru	-	Muroran	-	25/2/89	Eden
184	Konkar Victory	-	Kimitsu	-	11/1/89	Port Latta
185	Port Latta Maru	-	Wakayama(Japan)	-	6/1/88	Port Latta

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
186	M.V. Raicho	-	Sendai(Japan)	-	23/10/88	Launceston
187	Grace Taio	4	Iyomishima	-	29/11/88	Launceston
188	Konkar Victory	-	Chiba(Japan)	18/11/88	-	Port Latta
193	Maiorca	-	Ciquading	-	12/8/89	Port Latta
198	Bunga Srigading	fpt	Tomakomai(Japan)	14/8/89	22/8/89	Newcastle
199	Channel Fortune	db 2	Niihama(Japan)	26/9/89	12/10/89	Newcastle
201	Japan Platanus	-	Kimitsu	13/8/89	31/8/89	Newcastle
204	M.V. Nopal Cherry	stb top	Kobe	15/8/89	30/8/89	Geelong
205	M.V. Nopal Cherry	pt 3	Kobe	15/8/89	30/8/89	Geelong
206	M.V. Iran Jamal	stb w2	Bandar Abbas	-	13/8/89	Geelong
207	M.V. Iran Jamal	pt w2	Bandar Abbas	-	13/8/89	Geelong
208	M.V. Dooyang Elite	pt w3	Hereke(Turkey)	6/6/89	20/7/89	Geelong
209	M.V. Dooyang Elite	stb w3	Hereke	6/6/89	20/7/89	Geelong
211	Asean Victory	fpt	Shiogama(Japan)	14/10/89	5/11/89	Newcastle
215	Iron Wyalla	port 3	-	-	-	-
216	Iron Wyalla	stb 5	-	-	-	-
219	New Zealand Pacific	deep 1	-	-	-	-

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
220	M.V. Everbright	-	Muroran	1/11/89	15/11/89	Newcastle
223	Japan Platanus	-	Nagoya	-	13/10/89	Newcastle
226	Goyo Maru	w 4	Tubata(Japan)	8/11/89	24/11/89	Newcastle
227	Goyo Maru	w 5	Tubata	8/11/89	24/11/89	Newcastle
228	Interbulk Vision	w 4,5	Osaka	-	1/12/89	Newcastle
229	Pacific Taio	4	Iyomishima	-	17/11/89	Launceston
230	Daishowa Maru	-	Shimizu	-	11/11/89	Eden
400	Japan Platanus	w 5	Muroran	-	24/2/90	Mackay(Qld.)
401	Japan Platanus	w 5	Muroran	-	24/2/90	Mackay
402	Massey Phoenix	5	Pohang	-	8/11/89	Bunbury
403	Massey Phoenix	1	Pohang	-	8/11/89	Bunbury
404	Hokuetsu Ace*	fpt	Japan	-	8/12/89	Bunbury
405	Hokuetsu Ace*	4	Japan	-	8/12/89	Bunbury
406	Shirotae Maru	w 3	Muroran	26/1/90	15/2/90	Gladstone??
407	TNT Alltrans	stb w3	Bluff(New Zealand)	21/2/90	28/2/90	Gladstone
408	Global Peace	top 2	Pohang	12/2/90	1/3/90	Gladstone
409	TNT Alltrans	w	Bluff	21/2/90	28/2/90	Gladstone

* reballasted at sea

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
410	Global Peace	stb t3	Pohang	12/2/90	1/3/90	Gladstone
411	Marina Princesa	fore d	Lae??	31/1/90	19/2/90	Newcastle
412	Global Ling	w	Kawasaki	27/1/90	11/2/90	Gladstone
413	Lok Pragati	fpt	Wakayama,Japan	22/2/90	7/3/90	Gladstone
414	Fay Rouz IV	stb t2	Sagano Seri,India??	2/2/90	15/2/90	Gladstone
415	Rishikesh	stb db5	Nagoya	29/2/90	23/2/90	Gladstone
416	Global Ling	w 2	Kawasaki	27/1/90	11/2/90	Gladstone
417	World Light	top 3	Chiba	10/2/90	24/2/90	Gladstone
418	Rishikesh	db 5	Nagoya	9/2/90	23/2/90	Gladstone
419	World Light	stb t3	Chiba	10/2/90	24/2/90	Gladstone
420	Lok Pragati	w 2	Wakayama	22/2/90	7/3/90	Gladstone
421	Calliope Maru	stb db4	Nagoya	16/2/90	7/3/90	Gladstone
422	Chikubu Maru	w 5	Nagoya	9/2/90	21/2/90	Gladstone
423	Fay Rouz IV	top 1	Sagano Seri	2/2/90	15/2/90	Gladstone
424	Shirotae Maru	w 2	Muroran	26/1/90	15/2/90	Gladstone
425	Chikubu Maru	w 5	Nagoya	9/2/90	21/2/90	Gladstone
426	Spring Eagle	top 5	Yokohama	17/2/90	6/3/90	Gladstone

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
427	Spring Eagle	top 1	Yokohama	17/2/90	6/3/9	Gladstone
428	Calliope Maru	db 4	Nagoya	16/2/90	7/3/90	Gladstone
429	Sapporo Maru*	stb t7	Tomakomai	27/2/90??	4/3/90	Port Dalrymple??
430	Miyagi Maru	w 4	Matsushima(Japan)	1/3/90	13/3/90	Port Dalrymple
431	Ming Courage	top 3	Matuura(Japan)	28/2/90	13/3/90	Dalrymple Bay
432	Ming Courage	stb t3	Matuura	28/2/90	13/3/90	Dalrymple Bay
433	Shoho Maru	3	Takehara(Japan)	20/2/90	9/3/90	Dalrymple Bay
434	Shoho Maru	5	Takehara	20/2/90	9/3/90	Dalrymple Bay
435	Chennai Polivu	stb 4	India	-	1/3/90	Dalrymple Bay
436	Katori	w 3	Kaohsiung	16/2/90	9/3/90	Hay Point??
437	Sapporo Maru	top 7	Tomakomai	27/2/90	12/3/90	Port Dalrymple
438	Union Auckland	fpt	Wellington(N.Z.)	-	-	Mackay
439	Shoryca Maru	-	Fukuyama	22/2/90	10/3/90	Newcastle
440	Global Hope	stb t3	San Chong Pa(S.Korea)	8/2/90	22/2/90	Newcastle
441	Forest Queen	4	Shimizu	23/2/90	11/3/90	Newcastle
442	Mizukawa Maru	fpt	Mizushima(Japan)	20-24/1/90	14/2/90	Newcastle
443	Titus	w 2t	Hibikinada(Japan)	12/2/90	23/2/90	Newcastle

* reballasted at sea

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
444	Yamanaka Maru	top 4	Ube(Japan)	-	26/2/90	Newcastle
445	Stove Campbell	stb t6	Kawasaki	10/89	10/3/90	Newcastle
446	Pan Yard	top 5	Koseong(S. Korea)	8/2/90	24/2/90	Newcastle
447	Maersk Sam....??	w 4	Suao??	1/3/90	19/3/90	Newcastle
448	Chosan	-	Hong Kong	4/2/90	13/2/90	Port Hedland
449	Niizuru	stb w1	Mizushima	26/2/90	10/3/90	Port Hedland
450	Sincere Victory	stb db2	Suao	6/2/90	3/3/90	Port Hedland
451	Sincere Victory	stb db3	Suao	6/2/90	3/3/90	Port Hedland
452	Ken Ryu Maru	-	Fukuyama	9/2/90	23/2/90	Port Hedland
453	Chosan	-	Hong Kong	4/2/90	13/2/90	Port Hedland
454	Shensi	3	Mizushima	5/2/90	19/2/90	Port Hedland
455	Niizuru	w 1	Mizushima	26/2/90	10/3/90	Port Hedland
456	Sapphire Glory	db 1	-	-	8/3/90	Port Hedland
457	Sapphire Glory	top 3	-	-	8/3/90	Port Hedland
458	Sapphire Glory	top 3	-	-	6/3/90	Port Hedland
459	Sapphire Glory	top 3	-	-	6/3/90	Port Hedland
460	China Steel Innovator	stb 7	Kaohsiung	29/1/90	9/2/90	Port Hedland

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
461	China Steel Innovator	7	Kaohsiung	29/1/90	9/2/90	Port Hedland
462	Port Hedland Maru	1	Fukuyama	14/2/90	28/2/90	Port Hedland
463	Port Hedland Maru	3	Fukuyama	14/2/90	28/2/90	Port Hedland
464	Shensi	3	Mizushima	5/2/90	19/2/90	Port Hedland
465	Vivita	top 2	Haldia	27/2/90	21/3/90	Gladstone
466	Morning Camelia	top 6	Taichung(China)	2/3/90	19/3/90	Gladstone
467	Fortune-C	3	Kawasaki	28/2/90	18/3/90	Gladstone
468	Olga Topic	top 5	Isabel	2/3/90	16/3/90	Gladstone
469	Olga Topic	stb t5	Isabel	2/3/90	16/3/90	Gladstone
470	Enterprise	top 4	Yokahama	26/2/90	12/3/90	Gladstone
471	Kaii Maru	w 4	Kashima	1/3/90	15/3/90	Gladstone
472	Vivita	stb t2	Haldia	27/2/90	21/3/90	Gladstone
473	Ocean Trader	top 5	Kasado??	26/2/90	12/3/90	Gladstone
474	Sophia	stb t4	Kaohsiung	27/2/90	12/3/90	Gladstone
475	Akashi Maru	stb t5	Mizushima	1/3/90	17/3/90	Gladstone
476	Akashi Maru	top 5	Mizushima	1/3/90	17/3/90	Gladstone
477	Kii Maru	db 3	Kashima	1/3/90	15/3/90	Gladstone

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
478	Ikan Tongkol	stb t2	Mizushima	25/2/90	9/3/90	Gladstone
479	Ikan Tongkol	stb t5	Mizushima	25/2/90	9/3/90	Gladstone
480	Morning Camelia	top 6	Taichung	2/3/90	9/3/90	Gladstone
481	Fortune-C	stb t	Kawasaki	28/2/90	18/3/90	Gladstone
482	TNT Alltrans	2	Bluff	8/3/90	14/3/90	Gladstone
483	TNT Alltrans	1	Bluff	8/3/90	14/3/90	Gladstone
484	Enterprise	top 2	Yokohama	26/2/90	12/3/90	Gladstone
485	Ocean Trader	stb t4	Kasado	26/2/90	12/3/90	Gladstone
486	Sophia	top 4	Kaohsiung	27/2/90	13/3/90	Gladstone
487	Maria Angelicoussi -		Kuantan(China)	14/2/90	5/3/90	Port Kembla
488	Maria Angelicoussi	-	Kuantan	14/2/90	5/3/90	Port Kembla
489	Lauro	stb t3	Ube	3/1/90	13/3/90	Darwin(NT)
490	Lauro	stb t4	Ube	3/1/90	13/3/90	Darwin
491	Lauro	stb t3	Ube	3/1/90	13/3/90	Darwin
492	Union Rotoiti*	db 6	-	-	20/2/90	Sydney(NSW)
493	Union Rotoiti*	db 7	-	-	20/2/90	Sydney
494	Tone Maru	stb t3	Sasebo	5/3/90	30/3/90	Port Kembla

* reballasted Newcastle 8/2/90

Table 1. continued

Code	Vessel	Tank	Port of Origin	Departure date	Arrival Date	Port
495	Tone Maru	top 3	Sasebo	5/3/90	30/3/90	Port Kembla
496	World Eden	top 1	Nagoya	20-23/2/90	16/3/90	Port Kembla

Key
b : bottom
db : double bottom
fore d : fore deep
fpt : fore peak tank
pt : peak tank
stb : starboard
stb db : starboard, double bottom
stb t : starboard, topside
top : topside
w : wing

Table 2. Harbour Samples Tested

Code	Origin	Date
A	Smithton, Tas.	23/9/87
B	Tamar River, Tas.	10/9/87
C	Bathurst Harbour, Tas.	27/10/87
D	Nubeena, Tas.	14/10/87
E	Georges Bay, Tas.	11/7/87
F	Sullivan's Cove, Tas.	22/6/87
G	Devonport, Tas.	24/9/87
H	Port Esperance, WA	27/1/88
I	Deep Bay, Tas.	1/12/87
J	Hobson's Bay, Tas.	april'87
K	Triabunna, Tas.	27/8/87
L	Port Arthur, Tas.	13/10/87
E1	Fisherman Island	22/1/90
E2	Patrick's Wharf	22/1/90
E3	Fisherman Island	22/1/90
E4	Fisherman Island	24/1/90
E5	Fisherman Island	24/1/90
E6	Swing Basin	24/1/90
E7	Gladstone	16/1/90
E8	Gladstone	16/1/90
E9	Gladstone	16/1/90
E10	Gladstone	16/1/90
E11	Barney Point	16/1/90
E12	Barney Point	16/1/90
E13	Gladstone	16/1/90

Table 2. continued.

Code	Origin	Date
E14	Hay Point	17/1/90
E15	Hay Point	17/1/90
E16	Dalrymple Bay	17/1/90
E17	Dalrymple Bay	17/1/90
E18	Twofold Bay	17/11/89
E19	Twofold Bay	17/11/89
E20	Twofold Bay	17/11/89
E21	Twofold Bay	17/11/89
E22	Twofold Bay	17/11/89
E23	Twofold Bay	17/11/89
E24	Port Botany	16/11/89
E25	Port Botany	16/11/89
E26	Port Botany	16/11/89
E27	Port Botany	16/11/89
E28	Port Kembla	16/11/89
E29	Port Kembla	16/11/89
E30	Port Kembla	16/11/89
E31	Port Kembla	16/11/89
E32	Port Kembla	16/11/89
E33	Newcastle	15/11/89
E34	Newcastle	15/11/89
E35	Newcastle	15/11/89
E36	Newcastle	15/11/89
E37	Newcastle	15/11/89
E38	Sydney Harbour	16/11/89
E39	Sydney Harbour	16/11/89
E40	Sydney Harbour	16/11/89

Table 2. continued.

Code	Origin	Date
E41	Sydney Harbour	16/11/89
E42	Sydney Harbour	16/11/89
E43	Portland	12/12/89
E44	Portland	12/12/89
E45	Portland	12/12/89
E46	Portland	12/12/89
E47	Portland	12/12/89
E48	Portland	12/12/89
E49	Portland	12/12/89
E50	Portland	12/12/89
E51	Portland	12/12/89
E52	Portland	12/12/89
E53	Geelong	-
E54	Geelong	-
E55	Geelong	-
E56	Geelong	-
E57	Geelong	-
E58	Geelong	-
E59	Geelong	-
E60	Geelong	-
E61	Geelong	-
E62	Geelong	-

Table 3. Evaluation of trypticase medium +/- trypsin for the isolation of *C.botulinum* from ballast**

	C.botulinum Strain					Uninoculated
	A(21)	B(7723)	B(17)	E(Minn)	F(202)	controls
Ballast sample	429* 429+T	429* 429+T	439* 439+T	470* 470+T	414 414+T	414 429
			439 439+T	470 470+T	475# 475+T	439 470
					475 475+T	475

** Extracts from all inoculated samples caused the death of a mouse with symptoms typical of botulism.

Mice receiving extract from uninoculated samples survived.

+T Trypticase medium containing 0.1%w/v trypsin.

* Sample confirmed by neutralization tests with specific *C.botulinum* antiserum to contain botulinum toxin.

1/10 dilution of sample caused death of a mouse, mouse receiving a 1/100 dilution survived. Sample toxicity lost prior to completion of neutralisation tests.

Table 4. Comparison of anaerobic counts from nine ballast samples (counts expressed as colony forming units/ml ballast sediment)

Sample	RCA	RCA + Cycloserine*	RCA + egg yolk#
411	2.38×10^5	2.91×10^4	4.91×10^4
415	2.10×10^5	1.56×10^4	7.00×10^3
424	1.51×10^5	1.14×10^3	5.22×10^3
429	4.95×10^4	2.88×10^3	7.09×10^3
434	2.64×10^4	3.18×10^3	2.82×10^3
439	8.64×10^3	9.27×10^2	4.45×10^3
442	1.23×10^5	3.91×10^3	4.14×10^3
470	9.01×10^3	4.50×10^3	2.64×10^3
471	6.91×10^4	6.04×10^3	3.24×10^3

* Cycloserine 250μg/ml added to RCA

\# Egg yolk (50% solution of egg yolk in 0.75% saline) 10ml added to 90ml RCA

Table 5. Survival of *C.botulinum* type B spores inoculated into ballast and stored at 5, 10 ,15 and 20°C.

Counts from unheated ballast

°C	Day 0	Day 7	Day 14	Day 21	Day 28
U	8.6×10^3	4.9×10^3	4.4×10^3	5.4×10^3	2.4×10^3
5	6.0×10^5	7.9×10^5	7.8×10^5	1.3×10^6	1.4×10^6
10	3.4×10^5	7.0×10^5	8.1×10^5	1.4×10^6	1.2×10^6
15	8.3×10^5	6.2×10^5	6.6×10^5	1.2×10^6	1.5×10^6
20	2.6×10^5	2.2×10^4	5.1×10^5	1.3×10^6	1.1×10^6

Counts from ballast heated 65°C/30 min

°C	Day 0	Day 7	Day 14	Day 21	Day 28
U	NT	5.1×10^3	5.6×10^3	3.3×10^3	5.1×10^3
5	NT	8.0×10^5	6.7×10^5	1.0×10^6	8.8×10^5
10	NT	5.5×10^5	6.0×10^5	1.1×10^6	9.5×10^5
15	NT	5.4×10^5	3.5×10^5	1.0×10^6	9.0×10^5
20	NT	1.2×10^4	5.8×10^5	5.7×10^5	6.2×10^5

Table 6. Survival of *C.botulinum* type E spores inoculated into ballast and stored at 5, 10 ,15 and 20°C.

Counts from unheated ballast

°C	Day 0	Day 10	Day 19	Day 26	Day 33
U	3.8×10^4	1.1×10^4	2.6×10^4	4.4×10^4	2.5×10^4
5	3.7×10^4	2.3×10^5	3.7×10^4	4.4×10^4	7.5×10^4
10	3.6×10^4	1.2×10^5	1.5×10^5	1.5×10^5	2.1×10^4
15	4.3×10^4	1.9×10^5	2.2×10^5	5.0×10^4	4.5×10^4
20	1.6×10^5	1.6×10^5	1.4×10^5	5.5×10^4	3.6×10^4

Counts from ballast heated 65°C/30 min

°C	Day 0	Day 10	Day 19	Day 26	Day 33
U	3.8×10^4	4.4×10^4	5.7×10^4	4.8×10^4	3.1×10^4
5	1.2×10^5	1.4×10^5	5.6×10^4	1.4×10^5	7.3×10^4
10	2.1×10^5	1.5×10^5	3.6×10^4	1.2×10^5	1.2×10^5
15	2.1×10^5	1.8×10^5	6.0×10^4	3.7×10^5	9.9×10^4
20	2.1×10^5	1.0×10^5	4.8×10^4	1.6×10^5	5.5×10^4

Table 7. Confirmatory tests on samples initially causing death in mice.

Sample no.	Initial results	Time recorded (hrs.)*	Sample+buffer	Sample with antitoxin poly	A	B	C	D	E
C	+	30	-		-	-			-
E36	+,--	20							
416	+	21	--	--					
444	+	45	--	--					
461	+,-	1.5							
472	+,++	20	++	--	++	++	--	++	++

+ indicates death of a mouse
- indicates survival of the mouse
* Time at which the mouse was examined, not necessarily the time of death

APPENDIX

Methods used for the isolation and cultivation of *C.botulinum* and determination of *C.botulinum* toxin

Media

TPYGC (Trypticase, BBL 11921, 50g; Peptone, Oxoid, L37 5g; Yeast extract, Oxoid L21, 1g; Glucose, 4g; cysteine hydrochloride, 0.5g; Distilled water 1000ml). The basal medium was prepared without glucose and adjusted to pH level 7.0 then autoclaved at 121°C/15min. A 20% (w/vol) solution of glucose was prepared and steamed for 30min. 2ml was added aseptically to 100ml of basal medium immediately after cooling, prior to inoculation.

Reinforced Clostridial Media (RCM, Oxoid CM149) was used for routine subculture of the strains of *C.botulinum*. When cultures or spore crops were counted 2% agar was added to RCM broth to give a solid medium (RCA).

Cycloserine: a stock solution containing 250μg/ml was prepared in sterile distilled water. 1ml of antibiotic was added to 100ml RCA to give a final concentration of 2.5μg/ml agar.

Egg Yolk: equal quantities of fresh egg yolk and 0.85% sterile saline were mixed and stored at 4°C prior to use. 10ml egg yolk solution was added aseptically to 90ml molten RCA, tempered to 50°C.

Gelatin phosphate buffer: 0.4% (w/v) gelatin was prepared and sterilized at 121°C/15min. Phosphate buffer, pH 6.5 (Na_2HPO_4, 28.39g; KH_2PO_4, 27.218g; distilled water 1l) was prepared by dissolving the phosphates separately, they were then mixed and made up to the final volume before sterilizing at 121°C/15min. Gelatin and buffer were mixed in equal quantities immediately prior to use.

Trypsin: Dehydrated trypsin powder (CSL laboratories, Melbourne, Australia) was rehydrated according to the manufacturers instructions to give a 12.5% solution. One ml trypsin solution was then added to each 100ml of TPYGC medium to give a final concentration of 0.1% trypsin in the medium.

Detection of vegetative cells or spores of *C. botulinum*

Approximately 10g of each ballast or sediment sample was inoculated into freshly prepared TPYGC broth which was then incubated at 30°C for 48 - 72h. The culture filtrate was centrifuged and 0.5 ml aliquots injected into mice which were observed over a period of 4d for symptoms typical of *C.botulinum* intoxication. Presence of the toxin indicates growth (and therefore presence) of *C.botulinum*.

Confirmation of *C. botulinum* toxin

Mice injected with *C.botulinum* toxin show typical symptoms: initial rapid respiration followed by laboured, gasping respiration, pronounced "pinching of the waist" and finally death.

Culture filtrates causing death of one or both replicate mice were investigated further to confirm the presence of *C.botulinum* toxin. Aliqots of the sample were mixed with polyvalent *C.botulinum* antiserum prepared against *C.botulinum* types A, B, C, D, E and F. A control sample mixed with an equivalent volume of gelatin phosphate buffer was also prepared. Duplicate mice were injected with 0.5ml of each sample and observed over a 4d period for death and symptoms typical of botulism. Protection of the mice receiving the sample containing polyvalent antiserum and death of the mice receiving sample mixed with buffer indicates the presence of *C.botulinum* toxin. A similar procedure was then repeated using antisera specific to the individual types of *C. botulinum* in order to determine the type of *C.botulinum* present. Once the presence of *C.botulinum* toxin was confirmed attempts were made to isolate the organism.

Isolation of *C.botulinum*

The enrichment broth containing the inoculated ballast sample was streaked onto Reinforced Clostridial Agar (RCA, Oxoid CM149 plus 2% agar) and incubated anaerobically for 48h at 30°C. Colonies showing morphology typical of *C.botulinum* were examined microscopically for the presence of cells with spores typical of *C.botulinum*. Single colonies were then subcultured into fresh TPYGC broth and incubated for 48h at 30°C. The culture supernatant was then checked for the production of the specific type of *C.botulinum* toxin. Confirmation of the specific toxin indicated isolation of *C.botulinum*.

Preparation of Spore Crops

The following strains of psychrotolerant, nonproteolytic *C.botulinum* were used for inoculation of the ballast sediments: Type B, strain 17B, type E, 'Minneapolis'and type F, 202F (All strains were supplied by T A Roberts, AFRC, Institute of Food Research, Reading Laboratory, Shinfield, Reading, RG2 9AT, UK). All three strains were routinely subcultured in RCM, sporulation was attempted in two media, TPYGC broth incubated at 30°C and RCA incubated anaerobically at 30°C, broths and plates were examined every 2-3 days for the presence of spores.

Broths containing more than 50% spores were centrifuged at 3000g/10min, the supernatant was then decanted and cold sterile distilled water added. The spores were resuspended and centrifuged again. This process of centrifuging, decanting and resuspending in sterile distilled water was repeated 3 times to wash the spores. Growth on RCA showing more than 50% sporulation was washed off using cold sterile distilled water. The suspension was centrifuged and the spores washed as described above. The spores were then heated at 60°C/30min to destroy any vegetative cells present, centrifuged and resuspended in cold sterile distilled water then stored at 4°C until further use.

When sufficient spores had been obtained the suspensions were counted to estimate the number of spores present. Decimal dilutions were prepared using 0.1% peptone water and dilutions plated onto RCA using the pour plated method. Plates were incubated anaerobically at 30°C/2d and colonies counted.