Summary report

An introduction to *Clostridium botulinum* and its presence in UK soils and soil amendments

This report summarises the findings of three separate projects commissioned by WRAP to investigate the presence of the anaerobic bacterium *Clostridium botulinum* in soils and common soil amendments (including manures, slurries and composts). It also considers the impact of anaerobic digestion processes on *Clostridium botulinum*, and is expected to be of particular relevance to those using or considering the use of anaerobic digestates on grazed land.

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Front cover photography: Bandspread digestate application, *Clostridium botulinum* under scanning electron microscope, DNA ladder and digestate storage tank

Contents

Acknowledgements

This summary report is derived from three source documents, the authors of which are gratefully acknowledged:

- *C. botulinum in the environment*. Written by: Alison Rollett, Alan Lyne, Matthew Taylor and Brian Chambers (ADAS, Gleadthorpe, UK); Mike Peck, June Plowman, Jason Brunt, Andrew Carter, Duncan Gaskin, Martin Webb, Sandra Stringer (Institute of Food Research, Norwich, UK). WRAP project OMK002-008
- *Clostridium botulinum in Scottish and English AD systems.* Written by: E. Wachnicka, A.T. Carter, S.C. Stringer and M.W. Peck (Institute of Food Research, Norwich, UK); M. Taylor and A. Rollett (ADAS, Gleadthorpe, UK). WRAP project ORM001-001
- A review of the impacts of composting and anaerobic digestion processes on *Clostridium botulinum*. Written by: A Godley & R Smith (Ricardo-AEA, Harwell, UK). WRAP project OAV035-001

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1.0 Introduction

There is increasing agricultural demand for digestate and compost. This is due to recent dramatic volatility in the price and also availability of manufactured fertilisers, as a result of growing demand for food. Also, fibre digestate and compost are valuable sources of organic matter, which can improve soil physical and biological properties; namely water holding capacity, workability, structural stability etc. Notably, digestate and compost use can help to cut a farm's carbon footprint by reducing the need to apply manufactured fertilisers, which are either produced using fossil fuels (e.g. ammonium nitrate) or quarried and refined for transport/processing using fossil fuels (e.g. phosphate and potash). However, it is essential that the application of any organic material to land (agricultural or otherwise) is not harmful to the environment (i.e. soil, water and air quality) or human health.

As part of the drive to reduce the quantity of organic materials landfilled every year, the EU Landfill Directive requires a reduction of 35% in the quantity of biodegradable municipal waste sent to landfill by 2020 (compared with a 1995 baseline). The UK is also obliged to increase the quantity of renewable energy generated (the UK's share of the EU's binding target for renewable energy is 15% by 2020) and reduce greenhouse gas emissions (the Climate Change Act states UK emissions must be reduced by 80% by 2050). Two key processes in achieving the UK's legislative targets (for renewable energy and climate change) are anaerobic digestion (AD) and composting. It is estimated that just over 1 million tonnes of digestate and 2.8 million tonnes of compost are currently produced in the UK, with almost all the digestate and 60% of compost being recycled to agricultural land (WRAP, 2012). By 2020, it is predicted that there could be up to 5 million tonnes of food-based digestate produced (Defra and DECC, 2011), in addition to digestate produced from livestock manures and purpose-grown crops. Similarly, compost production may increase to around 4 million tonnes.

With the increase in popularity of anaerobic digestion as a waste treatment option, there has been interest in the bacterium which causes botulism (*Clostridium botulinum*). This bacterium requires anaerobic conditions to grow, and could be present in some or all of the starting materials used for AD (or composting). This report provides a summary of published literature on the occurrence and life-cycle of *Clostridium botulinum*, and presents recent data for its presence in samples of UK soils and soil amendments. Recent UK-specific data on the occurrence of the organism and its toxin at different points in commercial AD systems are also presented.

2.0 *Clostridium botulinum*

C. botulinum is a bacterial pathogen that causes botulism, a rare, but often fatal neuro-paralytic illness in human and animals.

There are seven main types of *C. botulinum* (Types A, B, C, D, E, F and H) based on serological (antigenic) properties of the toxins. Some of these toxin classes may be differentiated further into Sub-types, e.g. Peck (2009) indicates 5 Sub-type A (termed A1 to A5), 5 Sub-type B and 6 Sub-type E. Some strains also produce more than one toxin (Collins and East 1998, Peck 2009).

The different toxins show some specificity for causing disease in different hosts, with Types A, B, E and F predominantly causing human botulism, and Types C and D causing most cases of botulism in animals. However, this distinction is not absolute, as cases of botulism in cattle have been attributed to Types other than C and D (Notermans *et al.* 1979 and 1981).

The Type G strain has been re-classified as a different species (*C. argentinense*) (Suen *et al.*1988). Type H botulinum toxin has only recently been isolated (Barash and Arnon, 2014).

Strains of *C. botulinum* also differ in their physiological properties, with three recognised Groups (I to III) based on their proteolytic (ability to hydrolyse and utilise proteins as growth substrates) and saccharolytic (ability to hydrolyse and use polysaccharides as growth substrates) characteristics (Table 2-1).

For each group, there are differences in the extent to which they affect humans and animals, and the effects of temperature, pH, salinity and nutrients on growth rates, the formation and release of toxins, and the formation and germination of spores. For example, Group I and III strains grow between temperatures of about 10 and 50°C, whereas Group II strains grow within a lower temperature range of 3 and 45°C (Peck, 2009). These different characteristics can lead to uncertainty when trying to draw conclusions on the incidence and prevalence of *C. botulinum* from published reports.

Table 2-1 Phenotypic Groups of *Clostridium botulinum*

Properties	*C. botulinum* Group		
	I	II	III
Toxin types	A, B, F, H	B, E, F	C, D, C/D, D/C*
Major metabolic property	Proteolytic	Saccharolytic	Saccharolytic
Botulism in humans	Yes	Yes	No
Botulism in animals	Cattle/horses	Fish/possibly other animals	Cattle/horses, mink, foxes, other animals and birds

* Commonly strains of *C. botulinum* Group III form mosaic toxins described as either C/D or D/C that show features of both a type C neurotoxin and a type D neurotoxin

2.1.1 Spore production, germination and attenuation

The formation of spores in spore-forming bacteria allows them to enter a dormant form for survival under adverse conditions, such as starvation, toxic environments, temperature extremes, pressure, ultraviolet light and dessication – that would otherwise result in the death of vegetative cells.

The heat resistance of spores may vary, depending upon the environmental conditions under which they were produced, the age of the spores and the bacterial species producing the spores. The standard minimum food industry heat treatment "botulinum cook" for low-acid canned foods is intended to reduce the number of spores by a factor of 10^{12} (12-D). Peck *et al*. (2010) recommend a heat treatment of 90°C for 10 minutes for equivalent lethality, combined with storage at chill temperature for the safe production of minimally heated chilled foods to mitigate the risk of *C. botulinum*. Group I *C. botulinum* generally produce the most heat-resistant spores (Peck *et al.* 2010). Spores of the most resistant Group I strains with a 121°C D-value of 0.21 minutes (time required to reduce the spores by one order of magnitude [1-log reduction]) therefore require a treatment of 2.52 minutes (Peck 2009) at this temperature for food industry compliance (12-log reduction).

2.1.2 Toxin formation and attenuation

The production of toxin and its release from the cell by *C. botulinum* strains was discussed by Johnson and Bradshaw (2001) and by Peck (2009). Toxin production is complex and depends on the *C. botulinum* strain, different growth media constituents (such as nitrogen and carbon nutrition, and presence of heavy metals), the phase of growth and the environmental conditions. Several studies (Couesnon *et al,* 2006, Artin *et al.* 2008, Peck 2009) indicate toxin formation peaks during late exponential and early stationary phase of the bacterial growth cycle, i.e. the period when growth ceases due to the micro-organism

exhausting the supply of growth substrates – or changes to the environment (such as by lowering the pH) to conditions which do not favour growth. The release of toxin from the cell may differ between strains but can occur by cell lysis (possibly associated with spore release) or by secretion by intact cells (Peck 2009).

Botulinum toxins are heat labile and rapidly inactivated by temperatures greater than 80°C. The heat denaturation of botulinum neurotoxins was studied by Zhou *et al*. (2008) where the impact of different temperatures at 30 minutes exposure times on the toxicity and protein structure were monitored. The study concluded that heat treatments of at least 50°C resulted in significant (at least 3-log) decrease in toxin toxicity. Peck (2009) cites that 85°C for 5 minutes provides a 4-log reduction in active protein although it was indicated that the presence of different matrices can provide some protection.

3.0 Botulism

The primary route for botulism in animals is through consumption of the toxin; however, young mammals can also suffer from a form of infant botulism, e.g. shaker foal syndrome (Swerczek, 1980), and in rare cases wound botulism is possible. Liguori *et al.* (2008) reported a case of wound botulism in a 50 day old foal, where *C. botulinum* Type B was found to be present in lesions of the gastric mucosa.

3.1.1 Fish and avian botulism

Yule *et al.* (2006) reported the correlation between the presence of type E botulism in fish-eating birds with the consumption of affected fish, suggesting that the fish act as a vector to transport the toxin from the aquatic environment. The infected fish themselves showed prolonged moribund states and expressed changes in behaviour and pigmentation making it easier for birds to catch and consume affected fish, passing the botulism toxin to the avian population.

Outbreaks of avian botulism typically involve waterbirds or farmed birds such as poultry, and can involve thousands. Infant botulism in birds is also described (Miyazaki and Sakaguchi 1978). Avian outbreaks have been linked to wetland sediments (Zechmeister *et al.* 2005) and large outbreaks of Type C botulism can occur in wild waterfowl (Songer 1997) as happened in the Norfolk Broads in the summer of 1975 (Borland *et al.* 1977). A recognised concern is the use of poultry litter as manure on agricultural land and poultry manure contamination of home grown feeds such as silage – both of which represent possible routes of exposure to grazing livestock (DARDNI 2010, Section 3.1.2).

3.1.2 Cattle and other livestock

Botulism in cattle is a significant problem worldwide and indicated to be an increasing problem in the UK (VLA 2011). Lambert (2008) reports the results of a survey of 521 UK dairy and beef farms indicated that 58% had experienced animals dying for no apparent reason – an average of 2.6 animals per farm. Clostridial diseases were a possible cause of many of these, and where the death had been attributed to a clostridial disease 3% were attributed to botulism. Most cases are attributed to Type C and D toxin from consumption of contaminated silage and feed contaminated with infected animal material (Galey *et al.* 2000, Senturk and Cihan 2007). Cases of botulism have also been recorded for other *C. botulinum* toxins not normally considered a risk to cattle such as Type B strains. There has recently been identified a new form of cattle botulism (visceral botulism) which is not typically fatal but causes a general lack of health. The cause of this is mooted as exposure to *C. botulinum* spores which are able to colonise the lower parts of the intestine (Böhnel *et al.* 2001). *C. botulinum* Type C is also linked to Grass Sickness in horses as another form of toxico-infection, whilst pigs seem relatively resistant (ECA 2006).

3.1.2.1 Vaccinating livestock against Clostridial diseases

The wide range of toxin-forming clostridial species, accompanied by their wide natural distribution in soils and sediments mean that vaccination against clostridial disease in livestock is recommended at various times during the life of cattle (RUMA 2007). Two polyvalent vaccines are available, each providing protection against several clostridial diseases but not against *C. botulinum*:

- Vaccine 1 - *C. chauvoei, C. septicum, C. novyi*, and *C. tetani.*
- Vaccine 2 - *C. perfringens* (Types A, B, C and D), *C. chauvoei, C. septicum, C. novyi, C. tetani, C. sordelli and C. haemolyticum.*

Vaccines are available for *C. botulinum* types C and D, although these are only generally available under veterinary advice for use on cattle at risk of botulism under the Special Treatment Certificate (SCT) Scheme, e.g. in herds that have had cases of botulism (DARDNI 2010, VLA 2011). This vaccine is not thought to be widely used as a general precaution and at the time of writing had to be sourced from outside the EU (Australia or South Africa).

The potential wider use of the vaccine might mitigate risks where contaminated materials have been applied to grassland. In Germany use of the vaccine against *C. botulinum* is reported to alleviate the symptoms of visceral botulism in cattle.

4.0 *C. botulinum* in soils, soil amendments and the wider environment

4.1 Published literature

4.1.1 Soils

Clostridium botulinum is considered ubiquitous in soil throughout the world. The paper by Lúquez *et al.* (2005) investigated the occurrence of *C. botulinum* in Argentina, which was detected in between 4.4 and 38% of soils sampled from different regions. The same paper also referred to other studies indicating detection in soils at frequencies of 16.5% in Japan, 23.3% in Costa Rica and 24.3% in the USA.

Most of the information regarding the presence of *C. botulinum* in British soils is from studies that are more than 30 years old. Early studies in the 1920s-1940s cited by Smith and Young (1980) suggested that *C. botulinum* Type A or B was detected in 4-8% of British soil samples. Smith and Young (1980) reported results showing that of 174 UK soil samples, *C. botulinum* Type B strains were detected in 10 (5.7 %).

Smith and Milligan (1979) investigated soil samples collected from a redeveloped former Metropolitan (Caledonian) cattle market in London's Islington which operated between 1855 and 1939, housing 7,000 cattle, 35,000 sheep, 1,500 calves and 900 pigs. They showed that even after 40 years, 25% of the samples tested positive for *C. botulinum,* and no less than 4 strains (B, C, D and E) were found to be present. The authors suggested that the concentration of livestock at this location may have introduced the greater diversity of strain types and level of contamination in the soil, which has persisted since the closure of the market.

The mean spore count of Group I and II *C. botulinum* in Finnish soils was reported as between 50 to 1,050 spores/kg (Lindström 2009). Gale (2002) reported *C. botulinum* spore densities in soils ranging from 1-6 /kg for Great Britain and that the densities had been indicated as 2,500 /kg in potato fields in the Netherlands. The soil spore numbers are likely to be impacted by the addition of contaminated organic materials (such as manures) to soils. Notermans *et al.* (1981) reported an increase in soil spores densities from less than 0.2 /g to about 130 /g on a farm with an outbreak of botulism.

4.1.2 Sediments

Sediments are waterlogged and potentially anaerobic environments, and therefore likely to be suitable for the survival and growth of *C. botulinum*. The presence of *C. botulinum* has been determined in both fresh and marine sediments to high percentages and is implicated in botulism in wildfowl and birds (Kadlec 2002), and in fish.

Smith and Young (1980) summarised several UK studies indicating a higher level of detection in sediments than in soils:

- 72.5% of lakes and waterways in London (Smith and Moryson 1975)
- 35% of 554 mud samples from all parts of the UK (Smith *et al.* 1978)
- 98% of 45 samples from the Norfolk Broads (Borland *et al*. 1977)

The strains detected in these studies were mainly Type B, but Types C, D and E were also found, i.e. a greater diversity than in the soil studies. However, this might also reflect higher contamination levels, making detection easier. Hielm *et al.* (1996) using a PCR method detected Type E strains in 80% of sediment samples with spore counts ranging between 95 - 2710 (average 940) per kg - which are similar to soil levels.

Sediments from dredgings are applied to agricultural land with minimal treatment (excess water may be allowed to drain away prior to spreading) and would represent an existing source of *C. botulinum* for agricultural soils.

4.1.3 Feedstocks for anaerobic digestion and composting processes

Feedstocks for AD and composting processes can include food and garden wastes, manures/slurries and other animal by-products. Significant work has been carried out investigating the occurrence of *C. botulinum* in foods. As the outer surfaces of food such as vegetables are likely to have adhering soil and micro-organisms, and as these are typically removed as peelings which are discarded as waste, it is likely that household food wastes may retain and concentrate any associated *C. botulinum* contamination of the original food items.

4.1.3.1 Fish

Lalitha and Surendran (2002) found *C. botulinum* in 19% of fresh and 10% of cured fish samples in India. Among the *C. botulinum* types, A to D were prevalent in fresh fish, and types C and D were the prominent types found in cured products. In contrast, Hielm *et al.* (1996) using a PCR method did not detect *C. botulinum* in fish and fish intestinal samples from rainbow trout. This was despite the occurrence of Type E strains in 80% of sediment samples where the fish were taken. Sediment spore counts ranged between 95-2710 (average 940) spores per kg. Huss *et al.* (1974) however sampled 530 rainbow trout from four farms, showing that 64% of the fish tested positive for *C. botulinum* type E, with an estimated spore count of 340 to 5300 spores per kg.

4.1.3.2 Meat

Meat is considered to be potentially contaminated with *C. botulinum* from faecal and gut contents transferred during butchery after slaughtering. Lindström *et al.* (2006) cites a study detecting Group II *C. botulinum* in 36% of meat samples in Germany. Gale (2002) indicates detection in 4.2% of vacuum packed bacon samples.

4.1.3.3 Dairy products

The risk of direct contamination of milk from botulism infected cattle (and therefore dairy products such as cheese) is thought to be very low (ACMSF 2006 and 2009). Böhnel *et al.* 2005, however, detected the presence of Type B neurotoxin in milk from a cow affected by visceral (intestinal) botulism and also showing mastitis. Whilst most studies (noted by Böhnel

et al. 2005) in cows affected by acute botulinum test negative in milk, the study indicates the potential for the toxin to pass into milk.

4.1.3.4 Vegetables

Many cases of vegetable-related food botulism (Lindström *et al.* 2006) are from foods preserved in cans or oil. It is likely that these foods started from a low level of *C. botulinum* which grew during subsequent storage of the preserved food. Lindström also reports the detection of *C. botulinum* in some raw vegetables (4.3% of vegetable samples in an Italian study and 68% of potato peels from Swedish study). Therefore, the presence of *C. botulinum* in foods that may be eaten after minimal preparation is already likely. Braconnier *et al.* (2003) showed that growth of *C. botulinum* in pureed potato, broccoli and mushrooms occurred to sufficient high cell numbers ($\sim 10^8$) to produce detectable toxin under ideal laboratory conditions. Therefore growth of *C. botulinum* in contaminated vegetables stored under warm anoxic conditions might be possible.

4.1.4 Outputs from anaerobic digestion and composting processes

Bagge *et al*. (2009) studied the influence of the AD process on *Bacillus* spp. and *Clostridium* spp. numbers. Ninety-seven faecal samples collected from healthy cattle, 20 samples from slaughterhouse waste intended for biogas production and 60 samples from different stages in the AD process were analysed. *Bacillus* spp. and *Clostridium* spp. were quantified, the various species counted and subcultured, and identified by biochemical methods and by 16S ribosomal ribonucleic acid (rRNA) sequencing. The most commonly isolated *Clostridium* spp. was *C. perfringens*. Pathogenic clostridia such as *C. sordellii* were detected in manure and slaughterhouse waste before and after pasteurisation, but not after digestion. *Clostridium septicum* was found in slaughterhouse waste and *C. botulinum* was found before and after pasteurisation, but again not after digestion.

As an anaerobic organism, *C. botulinum* would not be expected to grow under aerobic composting conditions and during maturation or storage, unless small anaerobic microenvironments were present. However, the production of botulinum toxins in anaerobically stored compost has been demonstrated. Böhnel and Lube (2000) examined compost from household food waste bins and the potential health hazard to humans and animals from *C. botulinum.* Tests on 91 samples collected from compost plants showed 49 (54%) contained *C. botulinum* spores. Analysis for spores, vegetative cells and toxin on 73 samples indicated that 25% showed direct toxin production. However, actual numbers of spores and vegetative cells were not determined, so it is difficult to put these findings into context.

4.2 Recent data for spores in UK soils amended with organic material

4.2.1 Soils

Fifty-seven soil samples were taken from replicated field experimental sites throughout Britain (during the period January-February 2013) that had received (at least) three years of repeated organic material applications and associated ('background') samples, as part of the WRAP/Defra funded "Digestate and Compost Use in Agriculture" project (Table 4-1). The soil samples were tested for the presence of spores of Group I (proteolytic) *C. botulinum*, Group II (non-proteolytic) *C. botulinum*, and Group III *C. botulinum*. This was done by enrichment of 25 g (wet weight) of soil samples in microbiological growth medium, and then testing for the presence of the three Groups of *C. botulinum* by PCR detection of the botulinum neurotoxin genes.

Table 4-1 Soil sample characteristics

Test samples	Sample type	Site location	Land use	Soil texture classification
1	Untreated	Ayr (Scotland)	Grassland	Medium
2	Untreated			
3	Untreated			
4	Food-based digestate			
5	Food-based digestate			
6	Food-based digestate			
7	Manure-based digestate			
8	Manure-based digestate			
9	Manure-based digestate			
10	Green compost			
11	Green compost			
12	Green compost			
13	Green/food compost			
14	Green/food compost			
15	Green/food compost			
16	Livestock slurry			
17	Livestock slurry			
18	Livestock slurry			
19	Untreated	Lampeter (Wales)	Grassland	Medium (heavy)
20	Untreated			
21	Untreated			
22	Food-based digestate			
23	Food-based digestate			
24	Food-based digestate			
25	Green compost			
26	Green compost			
27	Green compost			
28	Green/food compost			
29	Green/food compost			
30	Green/food compost			
31	Livestock slurry			
32	Livestock slurry			

Test samples	Sample type	Site location	Land use	Soil texture classification
33	Livestock slurry			
34	Untreated	Aberdeen (Scotland)	Arable	Sandy/light
35	Untreated			
36	Untreated			
37	Food-based digestate			
38	Food-based digestate			
39	Food-based digestate			
40	Manure-based digestate			
41	Manure-based digestate			
42	Manure-based digestate			
43	Green compost			
44	Green compost			
45	Green compost			
46	Green/food compost			
47	Green/food compost			
48	Green/food compost			
49	Livestock slurry			
50	Livestock slurry			
51	Livestock slurry			
52	Untreated	Faringdon (England)	Arable	Heavy
53	Untreated			
54	Untreated			
55	Food-based digestate			
56	Food-based digestate			
57	Food-based digestate			

4.2.1.1 Testing procedures

As the three bacteria (Group I *C. botulinum*, Group II *C. botulinum* and Group III *C. botulinum*) are physiologically distinct, different test procedures were required for each. Extensive preliminary work was carried out in order to optimise the procedures. This included optimisation of both the enrichment procedures and the PCR tests. An Internal Amplification Control (IAC) was included in the PCR test, to demonstrate full operation of the PCR test.

The first series of tests were for the presence of spores of Group I (proteolytic) *C. botulinum* and Group III *C. botulinum*:

1. Soil samples (as received) were added to the enrichment medium, pasteurised by a heat treatment equivalent to 95°C for 10 minutes and then incubated at 37°C for 6 days.
2. Following a sub-culture for one day at 37°C, samples were tested for the presence of botulinum neurotoxin genes by PCR.
3. A multiplex PCR test for type A, B and F neurotoxin genes was used for Group I *C. botulinum*, and a multiplex PCR test for type C and D neurotoxin genes was used for Group III *C. botulinum*.

The limits of detection of Group I *C. botulinum* strains and Group III *C. botulinum* strains were each established by adding (spiking) known concentrations of spores to control bottles containing a 'pooled' (i.e. mixed) soil sample.

The second series of tests were for the presence of spores of Group II (non-proteolytic) *C. botulinum*:

1. Soil samples (as received) were added to the enrichment medium, pasteurised by a heat treatment equivalent to 65°C for 30 minutes and then incubated at 12°C for 8 days.
2. Following a 24 hour sub-culture at 20°C, samples were tested in a multiplex PCR for type B, E and F neurotoxin genes.

The limit of detection of this method was established by adding (spiking) known concentrations of spores to control bottles containing a 'pooled' soil sample.

4.2.1.2 Detection limits

In all PCR reactions, the IAC was positive indicating the assay was working correctly. Most probable number (MPN) estimates gave the following detection limits:

- 953 spores per gram (wet weight) of soil for Group I *C. botulinum*
- 10 spores per gram (wet weight) of soil for Group II *C. botulinum*
- 235 spores per gram (wet weight) of soil for Group III *C. botulinum*

4.2.1.3 Results

All 57 samples tested negative for the toxin genes of Group I (proteolytic) *C. botulinum* (types A, B and F), Group II (non-proteolytic) *C. botulinum* (types B, E and F) and Group III *C. botulinum* (types C and D) in the PCR tests. The IAC worked in all tests indicating a successful PCR test. This does not, however, mean that spores were necessarily absent from these soil samples, since they may be present at concentrations below the estimated detection limits.

4.2.2 Soil amendments

4.2.2.1 Sources of materials

Seventy five individual organic material samples were collected during spring 2013 and analysed for the presence of Group I (proteolytic) *C. botulinum*, Group II (non-proteolytic) *C. botulinum*, and Group III *C. botulinum*. The organic material samples were mainly collected as part of the WRAP/Defra funded "Digestate and Compost Use in Agriculture" project (OMK001-001) from throughout Britain. Additional samples were taken to ensure that the samples represented the range of organic materials commonly recycled to agricultural land, Table 4-2. For certain organic materials, namely food-based and manure-based digestate, repeat samples were taken from the same source to assess any effect that seasonality might have on the presence of *C. botulinum.* The organic materials were refrigerated at 4°C prior to analysis. *C. botulinum* presence in each sample was assessed by enrichment of duplicate 25 g samples in microbiological growth medium, and then testing for the presence of the three Groups of *C. botulinum* by PCR detection of the botulinum neurotoxin genes. In each case, the limit of detection of the tests was also determined.

Table 4-2 Organic material characteristics

Test sample	Sample type	Country of origin	Season sample taken*
1	Food-based digestate	England	Autumn
2			Winter
3			Spring
4			Spring
5			Summer
6	Food-based digestate	Wales	Summer
7			Autumn
8			Spring
9	Food-based digestate	England	Spring
10			Autumn
11	Food-based digestate	England	Autumn
12	Food-based digestate	England	Autumn
13			Spring
14	Food-based digestate	England	Winter
15			Winter
16	Food-based digestate	England	Summer
17			Autumn
18	Food-based digestate	England	Winter
19			Winter
20	Food-based digestate	England	Spring
21			Winter
22	Food-based digestate	England	Autumn
23			Autumn
24	Food-based digestate	England	Autumn
25			Autumn
26	Manure-based digestate	Scotland	Autumn
27			Spring
28	Manure-based digestate	England	Autumn
29			Spring
30			Summer
31			Spring
32	Manure-based digestate	England	Autumn
33			Spring
34			Autumn
35			Summer
36	Green/food compost	England	Autumn
37			Spring
38	Green/food compost	Scotland	Autumn

Test sample	Sample type	Country of origin	Season sample taken*
39			Spring
40	Green/food compost	England	Autumn
41			Spring
42	Green/food compost	Wales	Autumn
43			Spring
44	Green/food compost	England	Autumn
45			Spring
46	Green compost	Wales	Autumn
47			Spring
48	Green compost	England	Spring
49			Spring
50	Green compost	England	Winter
51			Winter
52	Green compost	Scotland	Spring
53			Autumn
54	Green compost	England	Autumn
55			Autumn
56	Livestock slurry	Wales	Spring
57	Livestock slurry	Scotland	Spring
58	Livestock slurry	Scotland	Spring
59	Livestock slurry	England	Autumn
60	Livestock slurry	England	Spring
61	Livestock slurry	England	Autumn
62	Livestock slurry	England	Autumn
63			Spring
64	Farmyard manure	Scotland	Autumn
65	Farmyard manure	England	Spring
66	Farmyard manure	England	Spring
67	Farmyard manure	England	Spring
68	Farmyard manure	England	Autumn
69	Farmyard manure	England	Autumn
70	Layer manure	England	Autumn
71	Layer manure	England	Spring
72	Layer manure	England	Spring
73	Layer manure	England	Spring
74	Broiler litter	England	Autumn
75	Broiler litter	England	Spring

*Spring (February-April); summer (May-July); autumn (August-October); winter (November-January).

4.2.2.2 Testing procedures

The testing procedures for the organic materials were the same as those described for soils (Section 4.2.1.1).

4.2.2.3 Detection limits

In all PCR reactions, the IAC was positive indicating the assay was working correctly. Most probable number (MPN) estimates gave the following detection limits:

- $9.2x10^4$ spores per gram (wet weight) of organic material for Group I *C. botulinum*
- 0.5 spores per gram (wet weight) of organic material for Group II *C. botulinum*
- $1.5x10^2$ spores per gram (wet weight) of organic material for Group III *C. botulinum*

4.2.2.4 Results

For each of the 75 materials (except sample 67), duplicate 25 g wet weight samples were tested. For 68 samples, both test bottles were negative for spores of Group I (proteolytic) *C. botulinum*, and since the detection limit was estimated as $9x10^4$ spores per gram (wet weight) of organic material, it is likely that if spores were present they were at a concentration of less than $9x10^4$ spores per gram. Spores of Group I (proteolytic) *C. botulinum* were detected in seven of the individual samples; all were positive for the type B toxin gene in the PCR test. For sample 75 (broiler litter) both duplicate bottles tested positive. For the six further organic material samples (32 – manure-based digestate; 37 – green/food compost; 39 – green/food compost; 46 – green compost; 51 – green compost; 56 – livestock slurry), one of the two replicates was positive for type B neurotoxin gene.

Each test sample was negative for the toxin genes of Group II (non-proteolytic) *C. botulinum* (types B, E and F) in the PCR test. Taking account of the control tests, this indicates that there were less than 0.5 spores of Group II *C. botulinum* per gram (wet weight) of organic material.

Each test sample was negative for the toxin genes of Group III *C. botulinum* (types C and D) in the PCR tests. Taking account of the control tests, this indicates that there were less than $1x10^2$ spores of Group III *C. botulinum* per gram (wet weight) of organic material.

It should be noted that the organic material samples were heterogeneous and probably had an uneven distribution of spores of both [i] all three Groups of *C. botulinum* and [ii] other bacteria that may inhibit growth of all three Groups of *C. botulinum* and impede their detection. Preliminary work clearly demonstrated that the growth of other competing bacteria was the major factor preventing growth of all three Groups of *C. botulinum*, which makes determination of the presence of spores of *C. botulinum* (all three Groups) and their detection very challenging in these matrices. Also, duplicate 25g samples of organic material were tested; which are relatively small sample sizes.

4.2.2.5 Conclusions

Based on published literature and the organic material/soil sample data obtained during this study, it can be concluded that *C. botulinum* risks from recycling digestate and compost to agricultural land are no greater than for livestock manures, and that – provided good practice is followed – risks to livestock would be negligible. In this context, good practice means spreading digestate with a bandspreader/shallow injector to minimise grass contamination, and that minimum three week no grazing periods follow both digestate and compost spreading.

4.3 Recent data for spores and toxins in Scottish and English AD systems

4.3.1 Sampling approach

A sampling programme was designed and implemented to provide a comprehensive snapshot of the presence and levels of viable spores and neurotoxins of *C. botulinum* Groups I, II and III in feedstocks and digestate products from AD facilities in Scotland and England that accept Animal By-Product (ABP) containing food wastes as feedstocks and are eligible for PAS110 certification (or are certified to PAS110).

Eighty samples were taken from twelve AD plants across Scotland and England, as detailed in Table 4-3. Each site was sampled twice in one day. All samples were taken by experienced scientific staff, using standard operating procedures based on Chambers *et al.* (2001b) and Defra (2013) guidelines. Specifically, each sample was derived by mixing ten sub-samples. All sampling equipment was cleaned with ethanol before and after sampling to ensure no cross-contamination. After collection, samples were stored and transported to ADAS Gleadthorpe in a refrigerated vehicle before storage at 4°C.

Due to the mixing and continuous nature of AD systems, it was not possible to accurately measure the effects of storage on *C. botulinum* toxin or spore numbers in 'fresh' or 'stored' samples by sampling from an individual AD plant, since the 'fill and draw' process 're-inoculates' the stored material many times a day. However, the impacts of storage were of particular interest, so – prior to storage – the whole digestate samples were split into two sub-samples; one sub-sample was stored at 4°C and the other at 25°C for approximately 1 month to investigate the effect on *C. botulinum* spore numbers and neurotoxins. The samples held at 25°C for one month are termed stored samples, whilst those held at 4°C throughout are termed whole digestates (Table 4-3).

All other samples were stored at 4°C prior to analysis.

4.3.2 Spores

4.3.2.1 Testing procedures

Each of the 80 samples of organic material was tested in duplicate using the following approach:

- 25 g (wet weight) of received sample was weighed into 500 ml Duran bottles and dried for 48 – 72 hours in an oven at 60°C.
- Dried samples were transferred to an anaerobic cabinet and allowed to deoxygenate overnight.
- Aliquots of 250 ml of anaerobic cooked meat medium (CMM) and 1 ml of trypsin (22.5 g of Difco 1:250/100 ml) were added to deoxygenated samples.
- Samples to be tested for presence of *C. botulinum* Group I and Group III were heated for 38 minutes at 95°C in a water bath (to provide an equivalent heat treatment to 95°C for 10 minutes in the sample). Samples to be tested for presence of *C. botulinum* Group II were heated for 47 minutes at 67°C (to provide an equivalent heat treatment to 65°C for 30 minutes in the sample).
- Pasteurised samples were then incubated at 37°C for 6 days (Group I and III) or at 12°C for 8 days (Group II).
- Incubation of these initial enrichment cultures was followed by a 24-hour sub-culture in TPGY (Tryptone, Peptone, Glucose, Yeast Extract) broth at 37°C (Group I and III) or at 20°C (Group II). This sub-culture (enrichment dilution) step was included to dilute out compounds previously found to inhibit the PCR test.
- Following the 24-hour incubation of the sub-cultures, DNA was extracted and tested using PCR tests for appropriate neurotoxin genes. Two different PCR tests were used: one for type A, B, E and F neurotoxin genes, a second for type C and D neurotoxin genes (including C/D and D/C mosaics).

Table 4-3 Sampling strategy of anaerobic digester plants across Scotland and England. Please note that the geographical locations of the plants are omitted to maintain their anonymity

Site	AD System				Sample types (and sample numbers)				
	Digestion temp[1]	Pasteurisation[2]		Digestion type[3]	Feedstock	Whole digestate	Fibre	Liquor	Stored (25°C)[4]
Plant 1	Mesophilic	Pre AD	71°C/1h	Wet	2 (1 & 13)	2 (25 & 37)	2 (77 & 79)	2 (73 & 75)	2 (49 & 61)
Plant 2			72°C/1h		2 (10 & 22)	2 (34 & 46)	-	-	2 (58 & 70)
Plant 3			71°C/1h		2 (3 & 15)	2 (27 & 39)	-	-	2 (51 & 63)
Plant 4			72°C/1h		2 (6 & 18)	2 (30 & 42)	-	-	2 (54 & 66)
Plant 5			75 – 80°C/1h		2 (7 & 19)	2 (31 & 43)	-	-	2 (55 & 67)
Plant 6		Post AD	70°C/1h		2 (12 & 24)	2 (36 & 48)	2 (78 & 80)	2 (74 & 76)	2 (60 & 72)
Plant 7			70°C/1 – 2h		2 (4 & 16)	2 (28 & 40)	-	-	2 (52 & 64)
Plant 8			71°C/70min		2 (5 & 17)	2 (29 & 41)	-	-	2 (53 & 65)
Plant 9			71°C/1h		2 (8 & 20)	2 (32 & 44)	-	-	2 (56 & 68)
Plant 10			72°C/1h		2 (9 & 21)	2 (33 & 45)	-	-	2 (57 & 69)
Plant 11			57°C/5h	Dry	2 (2 & 14)	2 (26 & 38)	-	-	2 (50 & 62)
Plant 12	Thermophilic	Pre AD	70°C/1h	Wet	2 (11 & 23)	2 (35 & 47)	-	-	2 (59 & 71)

1. Anaerobic digester operating temperature; mesophilic 30 – 40°C and thermophilic 40 – 50°C
2. Whether the process includes a pasteurisation stage before the digester (pre AD) or after (post AD)
3. Digester type is whether the anaerobic digester is wet (<15% dry matter) or dry (>20% dry matter)
4. Samples of Stored digestate were held at 25°C for approximately 1 month in 'mini' storage tanks at ADAS Gleadthorpe, while Whole digestate, Fibre and Liquor were stored at 4°C for this period.

4.3.2.2 Detection limits

The detection limits were estimated to be:

- $1x10^3$ spores/g for C. botulinum Group I
- <1 spore/g for C. botulinum Group II
- $3x10^2$ spores/g for C. botulinum Group III

4.3.2.3 Results

- For *C. botulinum* Group I
  - Seventy eight of the organic samples were negative. Based on an estimate of the detection limit, it was concluded that these samples contained less than $\approx 10^3$ spores/g (wet weight) of organic material.
  - Two of the samples were positive, which means that spores of *C. botulinum* Group I type B were present at more than $\approx 10^3$ spores/g (wet weight) of organic material.
- For *C. botulinum* Group II
  - Eighty duplicate 25g organic material samples were negative for spores of *C. botulinum* Group II. Based on the estimated detection limit, it was concluded that all 80 samples contained less than ≈ 1 spore/g (wet weight) of *C. botulinum* Group II.
- For *C. botulinum* Group III
  - Seventy six of the organic samples were negative for spores of *C. botulinum* Group III. Based on an estimate of the detection limit, it was concluded that these samples contained less than $\approx 10^2$ spores/g (wet weight) of organic material.
  - Four of the samples were positive, which means that spores of *C. botulinum* Group III type C/D were present at more than $\approx 10^2$ spores/g (wet weight) of organic material.

The presence of spores of these three Groups of *C. botulinum* below the detection limits cannot be ruled out.

4.3.3 Toxins

4.3.3.1 Testing procedures

Detection of *C. botulinum* neurotoxins type A, B, C, D and E in organic materials was conducted using a series of sandwich Enzyme-Linked Immunosorbent Assays (ELISAs). Four separate ELISAs were used. For detection of type A neurotoxin, samples were tested in an ELISA that used antibodies kindly provided by Dr. L. Stanker (USDA, Western Regional Research Laboratory, USA), while commercial antibodies (Metabiologics, Madison, WI, USA) were used in tests to detect type B and type E neurotoxin. These were similar to those described previously (Peck *et al.*, 2010). A commercial ELISA kit (Bio-X Diagnostics, Jemelle, Belgium) using antibodies developed by Dr. C. Brooks (AFBI Northern Ireland) was used for detection of type C/D neurotoxin, according the manufacturer's protocol.

Preliminary ELISA tests using a series of nine sample dilutions in 1% gelatine-phosphate buffer (1, 1:2, 1:5, 1:10, 1:25, 1:50, 1:75, 1:100, 1:500) for toxins A, B and E were conducted in order to determine the appropriate sample dilution. As a result of this, all prepared samples were tested at a final dilution of 1:10 in the type A, C/D and E ELISA, while all prepared samples were tested at a final dilution of 1:20 in the type B ELISA. At these dilutions the detection of toxin in the organic samples was found to be similar to that in gelatine-phosphate buffer (i.e. the organic samples did not inhibit toxin detection).

Each ELISA test plate consisted of duplicate sub-samples from each extract, a standard curve made from neurotoxin toxoid (an inactivated form of the toxin) in 1% gelatine-phosphate buffer (except ELISA for detection of type C/D toxins) and negative and positive control samples. For a negative control, a 1% gelatine-phosphate buffer was used. The positive controls were culture supernatants of strain ATCC 3502 (for type A ELISA), strain CDC 5900 (for type B ELISA), strain NCIB 10619 (for type C/D ELISA), and strain CB-K-24E (for type E ELISA).

The method used for the type A, B and E ELISAs determined (i) the presence of botulinum neurotoxin, and (ii) when present the concentration of neurotoxin. Samples were classified as above the detection limit, and positive for neurotoxin if their mean absorbance was greater than "mean absorbance of all AD samples + 2 x standard deviation of all AD samples", as used previously (Peck *et al.*, 2010). For those samples where neurotoxin appeared to be present, the concentration was calculated from the slope of the standard curve for neurotoxin toxoid.

A similar method was used for the type C/D ELISA. Samples were classified as positive if their absorbance was greater than "mean absorbance of all AD samples + 2 x standard deviation of all AD samples".

4.3.3.2 Detection limits

For the type A, type B and type E ELISAs, consistent results were obtained from the duplicate sub-samples, and also when the testing of samples was repeated. However, it proved very difficult to relate the measured absorbance to a toxin concentration. There were two major reasons for this: (i) there were no known negative control samples of organic materials; (ii) the very heterogeneous nature of the eighty samples (which varied from undigested feedstock to stored digestate) led to a significant variation in the ELISA absorbance. Together these issues made it difficult to distinguish a negative sample with a high natural background from a positive sample containing a small concentration of neurotoxin.

Taking the detection limit as the "mean absorbance of all AD samples + 2 x standard deviation of all AD samples", the detection limits for the three ELISAs were estimated as; 5 ng/ml for the type A ELISA, 9 ng/ml for the type B ELISA, and 4 ng/ml for the type E ELISA. [Note for information: 10 ng/ml equates to approximately 10^3 MLD_{50}/ml].

The type C/D ELISA detects both these neurotoxins and also the various mosaic forms, and does not distinguish between them. However, again it proved very difficult to distinguish positive/negative samples, for the reasons explained above. According to the kit suppliers, the detection limit is similar to that previously reported by Brooks *et al.* (2010, 2011), as 1.5 – 3.9 ng/ml. Taking account of the ten-times dilution of all samples, the detection limit is perhaps 20 ng/ml.

4.3.3.3 Results

Difficulties were experienced in interpreting the ELISA results; firstly from the lack of a "truly" negative control, and secondly from the heterogeneous nature of the eighty samples. This has made it difficult to distinguish a negative sample with a high natural background from samples containing a low quantity of neurotoxin.

No sample appeared to contain a large quantity of botulinum neurotoxin types A, B, C, D or E. Sample 60 appeared to be moderately positive for type A and B neurotoxin, and it may be that toxin formation had occurred during storage of the AD sample at 25°C although viable organisms capable of forming type A and B neurotoxin were not detected in this sample. Several other samples were "weakly positive/false positive" for type A, B or E neurotoxin. One sample was "weakly positive/false positive" for type C/D neurotoxin.

5.0 Summary

Clostridium botulinum is widely distributed in the environment, and can impact on both humans and animals via a number of routes. It may be argued that grazing livestock are at greatest risk of exposure to any additional risk caused by compost or digestate use, since they naturally ingest quantities of soil whilst grazing – and this soil may have been amended with compost and/or digestate (as well as other amendments such as poultry litter).

The *C. botulinum* spore results discussed in Sections 4.2.1.3, 4.2.2.4 and 4.3.2.3 are summarised in Table 5-1. In comparison with results for other common organic materials and soils with a known history of receiving those materials, the results show that possible risks associated with *C. botulinum* spores associated with the recycling of food-based digestate to agricultural land are similar to those for other organic materials (including livestock manures, composts and manure-based digestate), with the exception of broiler litter (for which the risks are higher).

Cattle are most susceptible to botulinum neurotoxins of type C and D, and Moeller *et al*. (2003) established the dose of type C toxin to clinical signs of botulism in cattle. Seven adult lactating Holstein dairy cows were given *C. botulinum* type C complex toxin at 0.125 ng kg^{-1}, 0.25 ng kg^{-1}, and 0.5 ng kg^{-1} and observed for seven days. Those cows given 0.125 and 0.25 ng kg^{-1} failed to develop clinical signs of botulism (posterior paresis) during the 7-day observation period. The three cows given the 0.5 ng kg^{-1} dose all developed clinical signs of botulism. The median toxic dose (MTD50) for *C. botulinum* type C toxin was estimated to be 0.39 ng kg^{-1} (Moeller *et al*., 2003). This makes lactating Holstein cows approximately thirteen-times more sensitive to botulinum type C toxin than a mouse (on a per kg basis). If it is assumed that a cow weighs 700 kg, then approximately 270 ng type C neurotoxin would be a median toxic dose.

In just one of the eighty tested samples (sample 77, a separated fibre digestate) was type C/D neurotoxin marginally above the detection limit (i.e. >20 ng/ml). This sample was only weakly positive, and is possibly a false positive. If it is assumed that the amount of type C/D neurotoxin in sample 77 was 30 ng/ml, then approximately 9 ml material would need to be consumed to give the median toxic dose to a 700 kg cow. However, this calculation needs to be viewed with extreme caution and should not be over-interpreted given the previous comments on the ELISA results. In reality, the commercial ELISA used in the present study does not allow a reasonable estimate of the amount of contaminated digestate required to be consumed to intoxicate a cow with type C botulinum toxin. Survival of the neurotoxin in the environment also needs to be considered when evaluating the risk presented, as does the impact of the statutory no graze/harvest intervals – which are intended to minimise the potential for direct contact between the applied material and subsequent grazing stock. Good practice for digestate application is to use low-emission approaches where the digestate is placed at the base of the grass sward or injected into soil beneath the sward.

Solely (or predominately) food-based digestate is unusual outside Britain so there is limited wider literature to refer to when classifying the risk from recycling food-based digestate to agricultural land. The levels of *C. botulinum* spores within food-based digestate have been shown to be similar to livestock manures. While there is no such comparable data available on the prevalence of *C. botulinum* neurotoxin in other organic materials or soils, food-based digestate has been produced and recycled to agricultural land (including a significant quantity of cut and grazed grassland) in Britain for more than a decade. Indeed, an AD plant has recycled over 50,000 cubic metres a year, almost exclusively to grassland since 2003, without any known negative effects on livestock.

Based on the literature review and organic material/soil sample data presented in in Sections 4.2.1.3, 4.2.2.4 and 4.3.2.3 it can be concluded that *C. botulinum* risks from recycling

digestate to agricultural land are no greater than for livestock manures, and that – provided good practice is followed – risks to livestock would be negligible. In this context, good practice means spreading digestate with a bandspreader/shallow injector to minimise grass contamination, and that minimum three week no grazing periods follow digestate spreading.

The wider context also suggests that it is appropriate to read these conclusions across to *C. botulinum* toxin, whereby the risk from recycling food-based digestate to agricultural land is actually no greater than for livestock manures (and lower than poultry litter), provided all legislation and good practice is followed. For all digestates this means complying with the statutory no grazing/harvest intervals, and the use of precision application techniques to minimise grass contamination. For fibre digestates this also means following normal good practice for spreading farmyard manures, whereby visible signs of digestate solids should have disappeared before allowing access to grazing stock (Chambers *et al.*, 2001).

Table 5-1 Summary of detection of viable spores of *C. botulinum* Groups I, II, and III in soils and soil amendments*

Organic material	Number of samples tested*	Number of positive samples		
		Clostridium botulinum		
		Group I	Group II	Group III
Food-based digestate	25	0	0	0
Manure-based digestate	10	1	0	0
Green/food compost	10	2	0	0
Green compost	10	2	0	0
Livestock slurry	8	1	0	0
Farmyard manure	6	0	0	0
Layer manure	4	0	0	0
Broiler litter	2	2	0	0
Soil (untreated)	12	0	0	0
Soil (food-based digestate)	12	0	0	0
Soil (manure-based digestate)	6	0	0	0
Soil (green compost)	9	0	0	0
Soil (green/food compost)	9	0	0	0
Soil (livestock slurry)	9	0	0	0
Food waste feedstock	24	1	0	0
Whole food-based digestate	24	0	0	1
Stored food-based digestate	24	1	0	3
Separated liquor	4	0	0	0
Separated fibre	4	0	0	0

*All samples analysed in duplicate 25g samples, except soils which were single 25g samples. All soils tested were from sites with a known history of application of the organic material listed

6.0 References

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