Watershed issues associated with *Clostridium botulinum*: A literature review

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ABSTRACT

Botulism the disease, the related organism (*Clostridium botulinum*) and toxin have gained renewed attention in these times of heightened homeland security and bioterrorism preparedness. Since *C. botulinum* is ubiquitous in nature, botulism outbreaks resulting from environmental exposure can be of concern to watershed managers and drinking water utilities. This paper reviews aspects of naturally occurring *C. botulinum* in light of concerns for source water watersheds. Information regarding sources and occurrence of botulism, *C. botulinum* and botulism toxins are discussed. Ecology and physiology of environmental *C. botulinum* and cycles of disease are reviewed. Finally, the effectiveness of water treatment and disinfection measures is discussed.

Key words | botulism, *Clostridium botulinum*, watershed

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BACKGROUND

Botulism the disease, botulinum toxin which causes the disease, and the bacterium which produces the toxin, *Clostridium botulinum*, have gained renewed attention in these times of homeland security and concerns about bioterrorism. *Clostridium botulinum* spores have been detected in soil samples collected from sites in drinking water watersheds. This is not surprising, as *C. botulinum* is ubiquitous in the natural environment. However, questions have been raised about the potential hazard of this organism to human health. In particular, is a water supply watershed at any unique risk that managers need to account for as they conduct watershed protection programs? This review focuses on the natural occurrence of *C. botulinum* and its implications for watershed management. Sources of the disease are discussed, and the ecology of the organism is presented in order to assess the potential for its presence in watersheds. The physiology of the organism explains how it manifests adverse effects. Lastly, the current control and disinfection measures are summarized.

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THE ORGANISM

Clostridium botulinum is a ubiquitous Gram-positive, spore-forming obligate anaerobic bacterium that primarily inhabits soil, dust and other organic matter (Pediatric Bulletin website 2002). *C. botulinum* may produce toxins that are among the most highly toxic biological products known (Whitlock & Buckley 1997). Toxin production occurs during multiplication of the vegetative form of the bacteria following spore germination. The vegetative form requires organic matter and a complete absence of oxygen in order to grow and produce toxin. Optimum growth occurs at 25°C (77°F). Toxin production is optimized in the pH range of 5.7 to 6.2 and depends on the protein content of the environment in which it is growing (Stringer *et al.* 1999). All kinds of animal proteins are suitable for promoting toxin production. An especially potent toxin is produced in bird, mammal and other invertebrate carcasses. There is also a lesser researched and less understood, but very important role of bacteriophages, viruses that infect

bacteria, in the production of certain toxin types. Recent research shows that bacteriophages determine whether the toxin will be produced during *C. botulinum* growth and multiplication stages (Eklund *et al.* 1987; Rocke 1993). Infant botulism is of public health concern and typically results from the ingestion of *C. botulinum* organisms that colonize the intestine, with subsequent multiplication and toxin production (Midura 1996).

Clostridia are generally soil bacteria, and they tend to persist in wetland soils. Under favorable environmental conditions, *C. botulinum* spores have been known to survive many decades, withstanding temperatures higher than 100°C (Bohnel 2002). In dust, water or plant material, spores may be transported large distances. *C. botulinum* spores can also subsist in foods that are incorrectly or minimally processed. A contaminated region may serve as an incubation area from which the pathogens are washed away by water or carried away by wind to nearby unaffected areas (CDC 1998; Smith & Sugyiama 1988). Thus, humans, farm animals and wildlife may be endangered in formerly unaffected areas. The organism and its spores are widely distributed in nature. They occur in both cultivated and forest soils; bottom sediments of streams, lakes, and coastal waters; in the intestinal tracts of fish and mammals; and in the gills and viscera of crabs and other shellfish (FDA website 2003). Household solid waste may also serve as a source of *C. botulinum*, and the bacteria or bacterial spores may be spread by flies (Bohnel & Lube 2000; Bohnel 2002).

THE DISEASE

Botulism is an acute neurological disease characterized by a paralytic illness that affects all species of animals (Galey *et al.* 2000; Bohnel 2002; Hannett *et al.* 2003). Paralysis is caused by the neurotoxin produced by the bacterium *Clostridium botulinum*. Eight botulinum neurotoxin (BoNT) types have been identified: Types A, B, C_1, C_2, D, E, F, and G (Rocke 1993; Rocke *et al.* 1998; Shapiro *et al.* 1998). Each type is unique in its geographic distribution and species susceptibility (Whitlock 1996). Types A, B, E and F cause human botulism. Types C and D cause most cases of botulism in animals. Type G has been detected in soils of Argentina, but no outbreaks involving it have been recorded (FDA website 2003).

Three major types of botulism are recognized today which include foodborne, wound and infant botulism. Infant botulism is typically caused by ingesting spores from sources such as dust, soils, water and honey. In the United States, an average of 110 cases of botulism are reported each year. Of these, approximately 25% are foodborne, 72% are infant botulism, and the rest are wound botulism (CDC 1995). No case studies of waterborne botulism outbreaks were specifically identified between 1975 and 1995 (CDC 1995).

Foodborne or classical botulism can affect humans or animals and is a severe type of food poisoning caused by preformed toxin in consumed food. Although the incidence of this disease is low (33 cases reported in 2001; CDC website 2003), it is cause for much concern because of its high mortality rate if not treated immediately or properly (FDA website 2003). About ten to thirty botulism outbreaks are reported annually in the United States, and most are associated with inadequately processed packaged foods which provide hospitable (moist, limited air, presence of organic matter) incubation environments for *C. botulinum* spores (FDA website 2003). In horses, foodborne botulism is termed forage poisoning and most often results from consumption of contaminated feed but can result from ingestion of contaminated natural vegetation (Whitlock & Buckley 1997; Schoenbaum *et al.* 2000). Forage poisoning most often is associated with contamination by the type B toxin of *C. botulinum* but has been associated with toxin types A and C sporadically (Whitlock 1996; Aldous 2003). In cattle, botulism caused by toxin types C and D is associated with consumption of feed containing dead animal carcasses (Whitlock & Williams 1999). It is estimated that fewer than 300 cases a year among horses occur in the continental United States (Oglesby 2003).

The onset of foodborne botulism symptoms is usually 18 to 36 hours after ingestion of the food containing the toxin, although cases have varied from 4 hours to 8 days. Early signs of intoxication consist of marked lassitude, weakness and vertigo, usually followed by double vision and progressive difficulty in speaking and swallowing. Other common symptoms include difficulty in breathing, weakness of other muscles, abdominal distention, and constipation (FDA website 2003).

Wound botulism occurs at the site of a wound, where both the growth of the organism and toxigenesis take place

(Midura 1996). The toxins then reach other parts of the body via the bloodstream. Wound botulism, when it occurs in horses, is generally caused by wounds resulting from injection or castration, or through umbilical infections in foals (Bernard *et al.* 1987). Although wound botulism rarely occurs in humans, some cases have been confirmed and were associated with illicit intravenous drug use (Whitlock & Buckley 1997). There were 23 reports of wound botulism in 2001, among which one death was reported. The median age of patients reported to have human wound botulism in 2001 was 41 (CDC website 2003).

Infant botulism results from the ingestion of spores of *C. botulinum* which germinate, multiply and produce botulinum toxin within the infant's large intestine (Pediatric Bulletin website 2002). More than 90% of the annually reported cases in the United States are from California, Utah, and Southeast Pennsylvania. This is a likely consequence of high concentrations of *C. botulinum* spores in the soil of these regions (Pediatric Bulletin website 2002). There were 112 cases of reported infant botulism in 2001. The median age of patients was fourteen weeks; one death was reported (CDC website 2003). The various potential environmental sources of these spores are soil, cistern water, dust and foods (FDA website 2003). Honey is the one dietary reservoir that has been linked to infant botulism by both laboratory and epidemiologic studies (Midura 1996; Cox & Hinkle 2002; Pediatric Bulletin website 2002). Infant botulism is now more widely recognized since its identification by health officials in 1976. Significantly more cases are now being confirmed, including cases reported in other countries (FDA website 2003).

The clinical features of infant botulism include constipation, poor feeding, weakness, hypotonia, dysphagia and in severe cases, flaccid paralysis and respiratory failure, usually occurring after a period of normal development. However, the clinical signs may range from asymptomatic "carriage" of infant botulism to severe forms of the disease which resemble sudden infant death syndrome (SIDS). Most infants suffer from moderate to severe forms of the disease and require hospitalization (Pediatric Bulletin website 2002). Physician awareness is vital for early recognition and treatment as more than 70 percent of these infants will require mechanical ventilation (Cox & Hinkle 2002). Cox and Hinkle (2002) acknowledge the controversial role of breast-feeding in infant botulism. It has been reported that 70 to 90 percent of infants with botulism were breast fed; however, breast-feeding may delay the clinical severity of the condition thus allowing for administration of proper medical attention before the botulism proves fatal (Golding *et al.* 1997). There is no evidence that breast-fed infants experience an increased severity of disease as compared to formula-fed infants. Breast feeding may lessen an infant's exposure to waterborne bacteria or bacterial spores as formula is typically prepared with water that is heated which may promote vegetative outgrowth of spores.

A fourth type of botulism, which is similar to infant botulism but is yet to be classified, has been associated with gastrointestinal infections in adults after broad-spectrum antibiotic treatment and either intestinal surgery or inflammatory bowel disease (Midura 1996). Reports in the medical literature propose that these procedures may have altered the intestine's interior thus allowing the colonization of *C. botulinum*. The clinical aspects of the disease are said to be similar to those of infant botulism (FDA website 2003). There was one case of adult colonization botulism reported in 2001 caused by type F toxin from which the patient survived (CDC website 2003).

All forms of botulism can occur in humans as well as in animals. The human disease does not differ fundamentally from that of animals in clinical features, ease of diagnosis, supportive laboratory testing, management, or therapeutic measures. However, the rarity of human botulism contrasts with the frequent occurrence of animal botulism. Animals may appear depressed, dull, reluctant to move and take food, and may show evidence of dropping food from the mouth, abdominal distension, a low grade colic or even vomiting. The disease is characterized by a flaccid paralysis marked by weakness, muscle tremors, stumbling and recumbency (Critchley 1991).

DIAGNOSIS AND TREATMENT

Diagnosis of botulism in humans is confirmed by the detection of *C. botulinum* or its toxin in the patient's stool. The toxin is isolated and identified using mouse lethality testing, with typing confirmed by neutralization of the toxin by specific sera. Usually testing must also be

performed to differentiate botulism from diseases with common symptoms (Pediatric Bulletin website 2002). Electromyogram (EMG) studies can support an early diagnosis. Potential source samples, such as dust, soil, honey, corn syrup or foods, should be collected for investigation (Cox & Hinkle 2002). Diagnosis of botulism in large animals requires one or more of the following: (1) demonstration of a preformed toxin in the patient's serum, gastrointestinal contents or in a wound; (2) demonstration of *C. botulinum* spores in the gastrointestinal contents and/or in feed materials; or (3) detection of an antibody response to *C. botulinum* in patients recovering from suspected botulism. Clinical signs of botulism may be evident, but definitive diagnosis and typing require the demonstration of preformed toxin in plasma or serum obtained from the affected animals as early in the clinical course as possible (Whitlock 1996).

In animals, the treatment of wound botulism involves removal of the infected tissue followed by treatment with antimicrobials to prevent further proliferation of *C. botulinum*. For all botulism cases, botulism antitoxin is administered, and meticulous nursing and nutritional support are necessary for full recovery (Bernard *et al.* 1987). A toxoid is available for *C. botulinum* type B that serves as a vaccine against botulism for horses. A three-initial-dose vaccination program is recommended followed by a single annual vaccination. Botulism toxoid is considered extremely efficacious when administered properly. In fact, occurrence of clinical botulism in fully immunized horses has not been reported. However, horses from an endemic area should be revaccinated annually. The effectiveness of various other toxoids as vaccines for animals is under continued scrutiny. In sensitive watershed areas, managers might promote annual vaccination of animals to prevent outbreaks and proliferation of the disease-causing bacterium.

Supportive care is the foundation of therapy for human botulism. Patients, particularly infants, must stay in an intensive care unit that can provide the necessary airway management, tube feedings, and physical therapy (Mygrant & Renaud 1994). Botulinum immuno globulin, a human-derived antitoxin, has been administered to infants and has greatly reduced the recovery time and the need for mechanical ventilation and tube feeding. However, close monitoring for a month after recuperation is necessary to prevent potential relapse of the disease (Cox & Hinkle 2002). It is universally recommended that corn syrup and honey not be fed to infants less than one year old to prevent the occurrence of infant botulism. To prevent risk of infant botulism from water exposure, parents should be encouraged to use boiled water to prepare foods intended for infant consumption. Close supervision of infants is also advised to prevent the infant from orally contracting *C. botulinum* spores in soil (Pediatric Bulletin website 2002).

SOURCES

The sources and prevalence of *Clostridium botulinum* are important to recognize in order to assess its potential presence in any watershed. *C. botulinum* has been detected in raw water storage areas, Finnish trout farms, fish and environmental samples from a coastal area in northern France, dust, wetland sediments and other sources (Sandler *et al.* 1998; Fach *et al.* 2002). A number of incidents of detection of the different types of *C. botulinum* in varying environments around the globe are summarized.

Soils and dusts have often been cited as a significant reservoir of *C. botulinum*. Type B organisms are common in the soils of the mid-Atlantic states. Two forms of type B toxin occur, a proteolytic toxin, which has maximal toxicity, and a nonproteolytic toxin, which must be catalyzed by trypsin to be fully activated. The proteolytic type B toxin occurs most commonly in the soils of the Mid-Atlantic States and Kentucky (Kelch *et al.* 2000).

Forage botulism or food-related botulism was discussed as an important pathway for agricultural animals. For example, serious outbreaks of type D botulism have occurred in beef cattle that were fed chicken litter (Hogg *et al.* 1990). Type C botulism in cattle has also been associated with feeding ensiled chicken litter (Hogg *et al.* 1990; Kelch *et al.* 2000). Most of the cattle and horse forage botulism outbreaks reported in the Eastern United States have been type B, often in association with consumption of contaminated forage (Aldous 2003). In the Netherlands, botulism has occurred in cattle that were fed wet brewer's grains (Kelch *et al.* 2000).

In another incidence of cattle botulism, the culprit was identified as contaminated hay. Twenty-two lactating

Holstein cattle in Tennessee had clinical signs of intoxication with preformed *Clostridium botulinum* toxin. The diagnosis of botulism by the ingestion of preformed *C. botulinum* type B toxin was made by eliminating other potential diagnoses. This was proven by finding *C. botulinum* type B spores in three bales of round bale barley haylage that were fed to these cattle and by isolating preformed type B toxin from one of the three bales. Haylage that is harvested green and encased in black plastic bags to facilitate fermentation, was presumably contaminated by the botulinum toxin when fermentation failed to produce enough acid to lower the pH to 4.5, the pH below which *C. botulinum* growth is inhibited. Fermentation of hay, leading to horse forage botulism, has also been reported (Wright & Kenney 2003). Thus, hay fermentation presents a potential problem for farmers who use round hay balers to produce haylage (Kelch *et al.* 2000). Watershed managers might choose to implement an education plan that would inform farmers of hay management practices that prevent the growth and spread of *C. botulinum* and its toxins.

The possible presence of *C. botulinum* spores in marine sediments and seawater with subsequent contamination of fish and other seafood are potential sources of human botulism (Glenn *et al.* 1998; Lalitha & Gopakumar 2000; Fach *et al.* 2002). Nonproteolytic strains of *C. botulinum*, mainly type E, can grow and produce toxin in fish products at a temperature as low as 5°C, and temperature abuse in food preservation can permit the growth and toxinogenesis of proteolytic *C. botulinum* strains (Hielm *et al.* 1998; Fach *et al.* 2002). Industrially processed, vacuum-packaged, hot-smoked salmanoids have been responsible for a cluster of outbreaks in northern Europe. The consumers' demand for reduced use of sodium salts and the vacuum packaging used to prolong shelf lives have apparently created high-risk botulinogenic products that are largely dependent on refrigeration for safety (Hielm *et al.* 1998). Fish and fish products preserved at 5°C for a long period or fermented and stored at room temperature are important and severe food poisoning hazards (Fach *et al.* 2002).

In 1998, Finland experienced its first recorded outbreak of botulism which was related to fish. The incriminated food was a hot-smoked vacuum-packaged whitefish that was produced in Finland but was exported to Germany, where two persons developed botulism after they ate the fish. Although the raw material had been imported from Canada, it was suspected that farmed fish and fish farm environments in Finland harbored numbers of *C. botulinum* type E spores. The rates of occurrence of *C. botulinum* type E in Finnish natural sediments and nonfarmed fish have been estimated to be 71% and 20%, respectively. A total of 333 samples were tested with a neurotoxin-specific PCR assay. *C. botulinum* type E was found in 95% of the trout farms investigated in this study: 68% of the farm sediment samples, 15% of the fish intestinal samples, and 5% of the fish skin samples were positive for *C. botulinum*. These findings indicate that (i) *C. botulinum* type E is an organism that has adapted to northern aquatic environments and occasionally contaminates animal or human food chains at random, and (ii) there is or has been extensive spread and exchange of *C. botulinum* type E strains in the northern temperate regions, for which the vehicle has not been determined (Hielm *et al.* 1998).

Sediments from wet environments can affect avian communities with certain feeding behaviors as well as fish communities. A nested PCR assay was developed for the detection of *C. botulinum* type C_1 toxin gene in sediments collected from wetlands where avian botulism outbreaks had or had not occurred. Sediment samples were collected during avian outbreaks at the following complexes: Klamath National Wildlife Refuge (NWR) in Willows, California; Sutter NWR in Willows, California; and Kulm Wetland Management District (WMD) in Kulm, North Dakota. The C_1 toxin gene was detected in 16 of 18 sample sites, demonstrating both the ubiquitous distribution of *C. botulinum* type C in wetland sediments and the sensitivity of the detection assay (Williamson *et al.* 1999). Between November 2000 and November 2002, a large outbreak of botulism among aquatic birds occurred in eastern Lake Erie. Approximately 7,000 bird deaths in New York State in 2002 have been attributed to this outbreak of botulism. Type E *C. botulinum* was present in the sediments of Lake Erie, large numbers of different bird and fish species were involved in this outbreak, and multiple genetically different strains of *C. botulinum* were involved (Hannett *et al.* 2003).

Aquatic birds can be a source in maintaining *Clostridium* contamination in wetland ecosystems. It has been demonstrated that avian species, specifically poultry but including waterfowl, can harbor *C. botulinum* spores in

their gastrointestinal tracts (Gophen *et al.* 1991). Species of birds affected included loons, ducks, gulls and eagles. The bacterial spores can then be shed in their feces. Most outbreaks of avian botulism are caused by type C botulism, as in the case in Israel where several thousand gulls died (Gophen *et al.* 1991). Outbreaks in the U.S. have been estimated to result in hundreds of thousands to millions of water bird deaths annually (Locke & Friend 1987). The wetland environment may provide conditions (pH, redox potential, organic matter levels) where bacterial spores can survive considerable lengths of time. In one study, historical occurrence of avian outbreaks of botulism, causing proliferation of the bacterium, influenced the risk of a future outbreak (Rocke & Samuel 1999). It has been noted that the affected bird carcasses could be a source of the *C. botulinum* organisms and preformed toxin in drinking water if bird kills are located near intakes.

Although the environmental characteristics that promote outbreaks are poorly understood, Rocke *et al.* (1999) found that in wetlands where botulism outbreaks had occurred, a greater percentage of organic matter in the sediment and lower redox potential in the water was measured, than in paired control wetlands. They also found that pH, redox potential, temperature and salinity measured just above the sediment-water interface were associated with the risk of botulism outbreaks in wetlands, but relations were complex, involving nonlinear and multivariate associations. Regression models indicated that the risk of botulism outbreaks increased when water pH was between 7.5 and 9.0, redox potential was negative, and water temperature was >20°C. Risk declined when redox potential increased (>100), water temperature decreased (10–15°C), pH was <7.5 or >9.0, or salinity was low (<2.0ppt). Management strategies to reduce losses from avian botulism may include assessing potential effects in the watershed using this predictive model (Rocke & Samuel 1999).

With respect to specific environmental and animal reservoirs, the pathways through which humans might contact *Clostridium* or its toxin are of concern. As discussed above, fish from contaminated environments can transmit the organism and/or preformed toxins to humans. In addition avian carcasses, during an outbreak, can be a source of spores and preformed toxin in water sources and surface waters, if not managed properly.

Dusts and honey are often cited as significant sources of the organism causing infant botulism. Between 1982 and 1997, 146 cases of infant botulism were diagnosed by the identification of botulinal toxin and *C. botulinum* in feces of infants in Argentina. The majority of infants requiring medical attention lived in suburban or rural areas. All strains isolated belonged to *C. botulinum* type A, consistent with the predominance of this type in the soils of Argentina (Fernandez *et al.* 1999). However, for most cases of infant botulism, the source of *C. botulinum* was not identified. Since the infection occurs in the intestinal tract, numerous food products which were either consumed by or would be consumed by infants were tested. Infant foods such as sugar, cereal, dried-milk formula, vitamin supplements and canned fruits and vegetables were not found to be significant sources of *C. botulinum*. Spores of *C. botulinum* are naturally present in some samples of honey. Studies have also implicated the natural environment as a source of *C. botulinum* spores. The organism causing specific infant botulism cases is usually the type found in the soil of the area where the illness occurs (Smith 1979). *C. botulinum* has been isolated from environmental samples such as yard soil and vacuum cleaner dust (Midura 1996). Should spores reach drinking water supplies from environmental sources, contaminated water used to prepare formula or cereals for consumption by infants could cause infant botulism.

Botulism cases specifically associated with natural drinking water exposure have not been documented. However, three drinking water treatment plants in the Netherlands were tested for their capacities to eliminate *C. botulinum* from the water after an outbreak of avian botulism within their watersheds. *C. botulinum* type B, C, D, and E were detected in mud samples in the source water areas of these treatment plants. In two plants, *C. botulinum* was only found in the early purification stages but, in the third plant, the bacteria were also detected on the slow sand-filters (the last step of purification). Their presence was limited, however, to the upper sand layers of the filters. *C. botulinum* was never detected in the filtered water in this investigation (Notermans *et al.* 1980). This demonstrates that water treatment processes are an effective barrier to transmission of *C. botulinum* spores, as discussed further below.

Concerns have been raised regarding old solid waste and agricultural waste areas as potential sources of *C. botulinum* and preformed toxin that could move into a water body. Both waste types are cited as possible ways to move the organism from environment to environment. Bio-waste recycling and the production and use of bio-compost are viewed as "green" waste reuse alternatives that could be included in sustainability planning. This practice is required by law in Germany (Bioabfallverordnung-BioAbfV 1998) and products can be exported to both other European countries and even outside Europe (Groot & Steenhof 1997). Quality control for these processes typically takes no specific consideration of pathogenic anaerobic spore formers such as *C. botulinum*. Microbial safety testing of such products in the U.S. typically involves prescribed use of certain treatment parameters. For example, composted biosolids meet quality and safety regulations based on the duration (days or hours) and extent of elevated temperature (40 °C or 55 °C) reached during the composting process (Soares *et al.* 1995). In one study, samples of marketed bio-compost in Germany were tested and results showed that about 50% of the tested samples contained *C. botulinum*. This demonstrates that the use of bio-compost could represent a potential health hazard to humans and animals, especially when spores accumulate in the environment (Bohnel & Lube 2000). Illegal garbage dumping in Israel was cited as one source of a gull outbreak of Type C botulism that killed several thousand birds (Gophen *et al.* 1991). The birds had foraged for food in the garbage and ingested spores. Requirements for daily cover at landfills in the US helps to minimize this pathway for wildlife contamination.

Overall, the ubiquitous nature of the organism requires that watershed managers and water utilities understand how the organism becomes a hazard and how to control those hazards. The sections below address these concerns.

PHYSIOLOGY

Clostridium are a genus of Gram-positive, pleomorphic rods that form spores and are widespread in the environment including soils (Holt *et al.* 1994). Bacterial spores are typically characterized by long environmental survival times and increased resistance to environmental stresses such as temperature fluctuations and desiccation when compared to vegetative cells (Madigan *et al.* 2003). Optimum growth temperatures for *Clostridium* species range from 10°C to 45°C. The significance of soil reservoirs of spores is evidenced by an investigation of nine cases of infant botulism in Argentina between 1995 and 1997 that were linked to the type A toxin, which was consistent with the types of *C. botulinum* found in the soils (Centorbi *et al.* 1999).

Of specific concern with *C. botulinum* in watershed management is understanding the factors that trigger spore germination and vegetative cell outgrowth to produce toxin from both soil reservoirs and spores mobilized to surface waters. *C. botulinum* cells themselves are not hazardous to mammals and birds; however, the endotoxin can be extremely hazardous. Much of the research regarding spore germination and vegetative cell outgrowth has focused on the food environment rather than the natural environment, since adult and animal botulism is most often associated with ingestion of contaminated foods (CDC website 2003; FDA website 2003).

The first step in transformation of a spore into a vegetative cell that produces toxin is germination. In one study, the researchers reported that once germination occurred, the vegetative cells would find suitable growth substrates, thus germination is the rate limiting step in toxin formation (Chea *et al.* 2000). Among the eight biotypes of *C. botulinum* are both proteolytic and non-proteolytic strains, and the characteristics of spore germination, vegetative outgrowth, and toxin production can vary (Hatheway 1993). Laboratory studies of endospore formation have demonstrated that the presence of certain gene clusters to signal germination are similar among spore forming genera such as *Bacillus* and *Clostridium* (Broussolle *et al.* 2002). Laboratory solutions containing L-alanine and sodium bicarbonate ($NaHCO_3$) were moderately effective in eliciting spore germination response, and the addition of L-lactate to the same solutions resulted in a strong response (Broussolle *et al.* 2002). In the same study, inosine or AGFK (asparagine, glucose, fructose, and potassium ion) did not stimulate spore germination. This was indicative of a *ger*A type operon involved in the spore germination of a number of *Clostridium* species.

Chea *et al.* (2000) reported that the time to germination was a function of temperature, pH, and sodium chloride

(NaCl) concentration. In addition, the mean time to growth for non-proteolytic *C. botulinum* in laboratory studies increased with temperature and pH, with the optimum temperature between 24°C and 27°C (Chea *et al.* 2000). In studies looking at temperatures from 2°C to 50°C, germination was most rapid at 50°C (Grecz & Arvay 1982). A significant lag in germination was reported at temperatures between 2°C and 4°C. Vegetative cell outgrowth was reported to occur at temperatures from 6°C to 41°C, with 32.5°C demonstrating an optimum.

Other researchers also found pH to be a significant factor in spore germination. For example, germination and toxin production was significant in vegetable juices with pH greater than or equal to 5.72, no toxin was produced at less than or equal to pH 4.99, and the extent of toxin formation varied between 5.72 and 4.99 depending on the vegetable juice (Stringer *et al.* 1999). In another study using vegetable juices, germination and toxin production were highest in juices with higher pHs ranging from 6.92 to 5.03 (Carlin & Peck 1995). The type of acidity was also demonstrated to affect spore germination, outgrowth and toxin production (Young-Perkins & Merson 1987; Wong *et al.* 1988; Carlin & Peck 1995). Significant spore outgrowth and toxin production was noted in protein suspensions adjusted to pH between 4.6 and 4.1 with hydrochloric acid. In contrast, suspensions adjusted with citric acid only, the test media with the lowest protein concentration and highest pH (4.5), proved toxic to mice (Young-Perkins & Merson 1987).

pH also plays an important role in toxin activity. It was demonstrated that progenitor toxins made up of neurotoxin and non-toxic components are protected from degeneration by stomach acids. When the progenitor toxins are then exposed to alkaline conditions, similar to those in the human intestines, the neurotoxin and non-toxic components dissociate, to result in the active toxin form (Fujinaga *et al.* 1994).

Several researchers have applied mathematical models to spore germination and growth (Chea *et al.* 2000; Zhao *et al.* 2003). These studies demonstrated an interaction of environmental conditions affecting spore germination, outgrowth and toxin formation. A significant finding was the possibility of spore signaling (Zhao *et al.* 2003). That is, germinating spores may be able to signal other spores that proper conditions exist for germination.

These physiological studies of *Clostridium botulinum* may provide some insights into cycles in watershed environments that contribute to the potential for botulism toxicity outbreaks. The decay of organic matter releasing L-alanine coupled with proper pH and temperature conditions could trigger spore germination and outgrowth. Acidic soil and water conditions may stimulate progenitor toxins to remain intact until ingested by animals. Additional research is needed to assess when these conditions may occur within a specific watershed, and animal access to surface waters should be appropriately restricted.

CONTROL

This section discusses the literature regarding land use level controls as well as drinking water treatment effectiveness. Controls may prevent spore germination and outgrowth in the environment. Control may inactivate spores in drinking waters, or may inactivate preformed toxin in drinking waters.

As indicated above, aquatic and wetland sediments can be a significant reservoir of *C. botulinum* that can be regenerated through fish and avian uptake, proliferation within the animal, and shedding with the feces. The Fish and Wildlife Service (Locke & Friend 1989) cites that fluctuations in water level and inundation of formerly dry areas promotes fly populations and *C. botulinum* perpetuation (Locke & Friend 1989). They recommend that animal carcasses, including birds and fish, during an outbreak, be burned rather than buried to mitigate the cycle. Other researchers have demonstrated that wetlands with emergent vegetation could result in 1.6 to 2.9 $\log_{10}$ reductions in *C. perfringens* spores from treated sewage (Barrett *et al.* 2001). Perhaps in areas of concern, best management practices (BMPs) could be constructed to retard hydraulic mobilization of *Clostridium* spores within watersheds. In an investigation of wetland soils, Sandler *et al.* (1998) demonstrated that a number of marsh soil species of *Bacillus* and *Streptococcus* produce *Clostridium* inhibitory proteins. They cite that these organisms in concert with pH, temperature, redox potential and types of organic matter present, all affect *Clostridium* germination and outgrowth. A strategy in the future could be developed to promote these *Clostridium* inhibitory species.

However, significantly more research is needed before specific recommendations can be made.

Once the *C. botulinum* spores reach a waterway, the next line of defense is to inactivate or kill the spores before they can germinate, outgrow, and form toxin or otherwise infect humans. Numerous researchers have looked at *C. perfringens* spores, a cousin to *C. botulinum*, and *B. subtilis* spores in treatment and disinfection studies. Although these studies have targeted bacterial spores as surrogates for the protozoan pathogens *Cryptosporidium* and *Giardia*, information about *Clostridium* inactivation may be gained.

C. botulinum spores may be physically removed from drinking water via coagulation, clarification (settling or floatation), and filtration. In a study of slow sand filters, Notermans *et al.* (1980) reported that spores of *C. sporogenes* would only penetrate to a depth of 5 cm after 30 days. *C. botulinum* types B, C, D, and E were found in the raw water storage area for another treatment plant. Rapid sand filters contained similar proportions of each *C. botulinum* type as the raw water storage area; however, no spores were detected after coagulation, nor were they found after final rapid or slow sand filters (Notermans *et al.* 1980).

Typical doses of chlorine used in drinking water treatment (around 2 mg/l residual) are not enough to inactivate bacterial spores. One study noted that concentrations of hypochlorite ions on the order of 12 to 18 mg/l can actually stimulate spore germination provided certain media additives were present (Foegeding & Busta 1983). Thus, much of the literature focuses on alternative disinfectants including ozone, chlorine dioxide, UV and mixed oxidants. Ozone was reported to effectively inactivate bacterial spores with a CT ranging from 1.7 to 6.3 (Facile *et al.* 2000). In another study it was demonstrated that a 1% survival rate with exposure to 1.5 mg/l ozone at pH 3 occurred (Foegeding 1985). However, treatment of drinking water at a pH of 3 is not practical. The authors note, that their work demonstrated the effectiveness of spore coats as a barrier to ozone at typical disinfection pH conditions.

Chlorine dioxide (ClO_2) has been shown to be effective at inactivating bacterial spores at doses that do not result in significant toxic by-product formation. In one study, *Bacillus* and *Clostridium* spores behaved similarly and demonstrated 5 $\log_{10}$ reductions with a CT of approximately 100 mg-min/l (Chauret *et al.* 2001). A study using *Bacillus subtilis* spores and a chlorine dioxide dose of 2.5 mg/l resulted in 0.5 to 2 $\log_{10}$ reductions in spores depending on the temperature, which was varied from 5.2°C to 23.2°C, respectively (Radziminski *et al.* 2002).

UV irradiation is gaining much attention for full-scale treatment to reduce disinfection by-product formation. It has also been demonstrated to be much more effective at inactivating bacterial spores and protozoan pathogens than chlorine (Shin *et al.* 2001). In one study using *C. perfringens* spores, UV doses from 2.5 to 12.5 mJ/cm^2 were not demonstrated to result in more than 0.5 $\log_{10}$ reduction in viable spores (Hill *et al.* 2002).

Mixed oxidants is an emerging disinfection technology where an electric current is passed through a sodium chloride brine solution to produce the oxidants. In one study of *C. perfringens* as a protozoan surrogate, 5 mg/l of mixed oxidants over a 4 hour exposure was able to achieve greater than 3 $\log_{10}$ reductions when compared to 1.4 $\log_{10}$ for chlorine (Venczel *et al.* 1997). Another study of *C. perfringens* as a protozoan surrogate demonstrated that 10 to 13 mg/l mixed oxidants for a contact time of 90 minutes would achieve a 2.5 $\log_{10}$ reduction (Casteel *et al.* 2000).

In addition to spore transmission through water, transmission of preformed toxin is of concern. A limited number of studies have been conducted on the treatment of botulinum toxin. Markarjan *et al.* (1960) demonstrated that conventional chlorine residuals of 1-2 mg/l were ineffectual at inactivating botulinum toxin. However, if chlorine was raised to 9.6 to 13.7 mg/l with a contact time of 30 to 40 minutes, the toxin levels tested were completely inactivated. Boiling for 10 to 15 minutes, and a long contact time with activated carbon were also demonstrated to be effective. A more contemporary study noted that for toxin type E, a 20 minute treatment with ozone at a residual of 2.3 to 8.5 ppm was effective at inactivating $2x10^4$ MU/ml of toxin (Graikoski *et al.* 1985). The researchers also state that a more plausible water concentration would be $2x10^3$ MU/ml of toxin which would only require 1 minute of ozone with a residual of 0.81 ppm for inactivation. Such control measures are not practical for a utility balancing microbial control measures with disinfection by-product regulations to keep within economic constraints. However, in emergency situations, increasing chlorine or ozone doses to inactivate preformed toxin may be possible.

SUMMARY

It appears that *C. botulinum* spores naturally occur in soils and sediments with specific strains predominating in different geographical regions. In fact, they have been described to be ubiquitous. However, considering current source water protection practices in the U.S., threats of waterborne botulism outbreaks among human populations is low. Watershed managers need to be aware of threats within their watersheds such as animal agriculture vulnerability to forage botulism and outbreaks of avian and fish botulism. Threats of forage botulism can be minimized by education of farmers regarding hay and feed management practices. Threats to source waters from wildlife outbreaks can be minimized by proper detection and carcass management when avian and fish outbreaks occur. Conventional treatment processes have been demonstrated to be adequately effective at removing *Clostridium* spores from drinking water. In the case of intentional release of botulism organisms to a water supply, vigilant monitoring of treatment to remove spores is recommended. In case of contamination, intentional or environmental, with preformed toxin, increasing disinfection CTs to emergency levels can effectively inactivate the toxins.

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