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Prevalence of toxin-producing *Clostridium botulinum* associated with the macroalga *Cladophora* in three Great Lakes: Growth and management

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HIGHLIGHTS

- 96% of Great Lakes *Cladophora* mats contained *C. botulinum* type E in 2012.
- *C. botulinum* in *Cladophora* mats was100 fold greater than in sand or water.
- Steam treatment was effective in eliminating *C. botulinum* in *Cladophora* mats.

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ABSTRACT

The reemergence of avian botulism caused by *Clostridium botulinum* type E has been observed across the Great Lakes in recent years. Evidence suggests an association between the nuisance algae, *Cladophora* spp., and *C. botulinum* in nearshore areas of the Great Lakes. However, the nature of the association between *Cladophora* and *C. botulinum* is not fully understood due, in part, to the complex food web interactions in this disease etiology. In this study, we extensively evaluated their association by quantitatively examining population size and serotypes of *C. botulinum* in algal mats collected from wide geographic areas in lakes Michigan, Ontario, and Erie in 2011–2012 and comparing them with frequencies in other matrices such as sand and water. A high prevalence (96%) of *C. botulinum* type E was observed in *Cladophora* mats collected from shorelines of the Great Lakes in 2012. Among the algae samples containing detectable *C. botulinum*, the population size of *C. Botulinum* type E was 10^0–10^4 MPN/g dried algae, which was much greater (up to 10^3 fold) than that found in sand or the water column, indicating that *Cladophora* mats are sources of this pathogen. Mouse toxinantitoxin bioassays confirmed that the putative *C. botulinum* belonged to the type E serotype. Steam treatment was effective in reducing or eliminating *C. botulinum* type E viable cells in *Cladophora* mats, thereby breaking the potential transmission route of toxin up to the food chain. Consequently, our data suggest that steam treatment incorporated with a beach cleaning machine may be an effective treatment of *Cladophora*-borne *C. botulinum* and may reduce bird mortality and human health risks.

1. Introduction

Avian botulism is a paralytic disease of birds caused by neurotoxins produced by the naturally occurring anaerobe, *Clostridium botulinum*. This has been recognized as a major cause of mortality of birds in the Great Lakes (Brand et al., 1988; USGS National Wildlife Disease Center, 2008; Zuccarion-Crowe, 2009; US EPA Great Lakes National Program Office, 2012). The outbreaks were first reported in this region in 1963 (Kaufmann and Fay, 1964) and often occur on a yearly cycle. Botulism neurotoxin types C and E are primarily responsible for the die-off of waterfowl and fish-eating birds. In recent years, a re-emergence of type E botulism has been observed across the Great Lakes basin, with alarming increases in bird mortalities in the lower Great Lakes (USGS National Wildlife Disease Center, 2008; Byappanahalli and Whitman, 2009; Zuccarion-Crowe, 2009). While several studies have detected the presence of *C. botulinum* in the environment (Perez-Fuentetaja et al., 2011; Hannett et al., 2011; Chun et al., 2013; Espelund and Klaveness, 2014), and attempted to postulate how the toxin reaches birds (Byappanahalli and Whitman, 2009; Perez-Fuentetaja et al., 2006, 2011), there have been limited studies on environmental sources and potential transmission routes of *C. botulinum*.

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It has been postulated that recent bird death outbreaks may be a consequence of ecosystem alternations including water conditions (e.g. temperature, clarity, and water levels) (Lafrancois et al., 2011), the presence of invasive species such as invasive dreissenid mussels and round gobies (Getchell and Bowser, 2006), and the extensive growth of the nuisance macroalga *Cladophora* in this region (Byappanahalli and Whitman, 2009; Chun et al., 2013). Some of these factors are interconnected and contribute to the production of botulism toxins. For example, invasive mussels, which are postulated as sources of *C. botulinum* themselves, are thought to promote the growth of *Cladophora* by providing attachment sites making the lake water clearer for better light penetration (Depew et al., 2011). Algal thalli and exudates are rich in nutrients and are capable of supporting a variety of macro- and microorganisms, such as epiphytes, protozoa, rotifers, macroflora, and numerous bacteria (Taft, 1975; Stevenson and Stoermer, 1982; Chilton et al., 1986; Byappanahalli et al., 2003; Ishii et al., 2006). Decaying algae provide suitable low-oxygen and nutrient-rich habitats that promote the growth of a myriad of microorganisms, including *C. botulinum* (Smith and Sugiyama, 1988).

Cladophora has been hypothesized to be as a possible carrier transporting the toxin up the food chain via fish directly, or via several intermediate vectors, to fish and then to birds (Byappanahalli and Whitman, 2009; Perez-Fuentetaja et al., 2006, 2011). Thus, a high incidence of botulism in shoreline birds at Sleeping Bear Dunes National Lakeshore (SLBE) in Lake Michigan appears correlated with *Cladophora* accumulations. Byappanahalli and Whitman detected the botulism toxin type E gene (*bont/E*) in *Cladophora* mats from three beaches at SLBE (Byappanahalli and Whitman, 2009) but the study only examined the small number of *Cladophora* samples, did not test for toxin production and lacked information concerning the population size of *C. botulinum* in the algal mats examined. In our previous study, we determined the relative abundance and types of *C. botulinum* in 53 *Cladophora* mats collected from several geographic areas in Lake Michigan and Lake Ontario in 2011 (Chun et al., 2013). We reported that ~76% of the algal mats (39 of 53) from shorelines of the Great Lakes contained the *bont/E* gene, and the population density of *C. botulinum* approached 15,000 most probable number (MPN) per gram of dried algae. Furthermore, *C. botulinum* type E was present on *Cladophora* mats as viable/vegetative cells, suggesting that *C. botulinum* spores become incorporated into *Cladophora* from sediment, and subsequently germinate into actively growing and toxin-producing cells. Our findings of a high incidence of *C. botulinum* type E gene in nearshore *Cladophora* mats as vegetative cells lend support to the hypothesis that algae play an important role in type E disease outbreaks, primarily as an environmental source of the pathogen (spores, vegetative cells, or toxin) and part of the food web interactions. Along these lines, we also found that *C. botulinum* type E was present in quagga muscles (Perez-Fuentetaja et al., 2006), possibly also leading to the death of muscle-eating birds. While compelling, our findings were based on only one year of data and two Great Lakes.

In this study, we further examined population size and serotypes of *C. botulinum* associated with *Cladophora* mats found in a wide geographic area in Lakes Michigan, Ontario, and Erie in 2012. This region had an ~2 month early arrival of summer, with *Cladophora* accumulation observed in March and thousands of botulism-afflicted birds found dead on Lake Michigan beaches. We also compared the population density of *C. botulinum* type E in *Cladophora* with those in other matrices such as water and beach sand. Finally, we present the effect of several treatments on inhibition of *C. botulinum* type E in *Cladophora* mat as a potential management practice that can limit proliferation of *C. botulinum* in *Cladophora* mats and on beaches to potentially mitigate the impact of avian botulism in the Great Lakes. This may allow us to potentially break the transmission route and eventually reduce bird mortality and human health risks associated with *Cladophora* on Great Lakes beaches and waterways.

2. Experimental section

2.1. Site description and sampling

Cladophora, water, and beach sand samples were collected from shoreline and beaches in Lake Michigan, Lake Ontario and Lake Erie monthly during June–September in 2012. The study sites include shorelines along SLBE (Empire, MI), Door County, WI, the City of Racine, WI, Porter and Lake Counties along southern Indiana (Indiana Dunes National Lakeshores; IDNL) on Lake Michigan, Hamlin Beach State Park, NY in Lake Ontario, and Cuyahoga County, OH in Lake Erie (Fig. 1). Three replicate samples (150 g) of free floating *Cladophora* were obtained from two locations at each sampling site. In instances, the accumulation of *Cladophora* was insufficient to obtain two samples from each site, in this case, we collected the samples from only one location at each sampling site. The samples consisted of detached, free floating algal matter in nearshore waters, which have not yet washed-up onto the dry beach. Nearshore water samples (250 mL) below floating algal matter and wet beach sand (~100 g) from the swash zone were collected in triplicate. All samples were collected by gloved hands, placed in Whirl-Pak bags or PTFE bottles, and transported to the laboratory on ice at 4 °C. The samples were immediately shipped to the University of Minnesota and were analyzed within 24 h of collection. An axenic culture of *Pithophora* (a filamentous green alga) grown in the laboratory was used as negative control since there is no contamination with any clostridia.

2.2. MPN-PCR analyses

Five-tube MPN analyses (Alexander, 1982) were used to identify types of *C. botulinum* and to quantify their population density in *Cladophora*, water, and sand samples as previously reported (Chun et al., 2013). Briefly, wet algae (50 g) were pooled from three replicates. Extraneous plant materials and benthic invertebrates were removed from *Cladophora* samples using sterilized tweezers. Samples were homogenized with 100 mL of phosphate-buffered water (PBW, pH 6.8) in a laboratory blender using a sterile polypropylene container (500 mL; Waring™) under N_2 gas. Two subsamples (10 mL) were taken from the algal homogenates for total and spore counts. A subsample for total counts was used for MPN analysis and a separate subsample was used for quantification of spores following treatment at 80 °C for 10 min (Dowell and Hawkins, 1974). Aliquots of both the algal homogenates with and without heat treatment were serially diluted ten-fold, three times, in PBW. Aliquots (1 mL) of all subsequent dilutions were added to 9 mL of pre-reduced trypticase-peptone-glucose yeast extract (TPGY) broth as described (Hielm et al., 1996). The anaerobic culture tubes were incubated at 26 °C for 5 d after flushing with N_2 gas. After incubation, 2 mL aliquots of each tube were shaken vigorously and cells were pelleted by centrifugation and stored frozen at −20 °C until used genomic DNA extraction. A portion of the algal homogenate was placed in an oven overnight to measure the algal matter dry mass.

Water samples were filtered onto 0.22 μm nitrocellulose filters (Millipore; Billerica, MA) and microbial cells trapped on filters were extracted by agitation for 3 min in a 5 mL pyrophosphate buffer, pH 7.0. For beach sand, bacteria were elutriated by agitating 20 g subsamples of sand in sterile milk dilution bottles with 50 mL sterile ammonium phosphate solution containing 0.01% gelatin as previously described (Ishii et al., 2006). Supernatants from sand samples were filtered through 0.22 μm filters and cells were extracted from filters in the same manner as were water samples. Aliquots were sequentially diluted and used for three-tube MPN analyses as described above.

Genomic DNA from cell pellets was directly extracted using the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) as per the manufacturer's protocol. The microbead tubes with pellets were incubated at 80 °C before 10-min-vortexing (lysis process) to increase yield and detoxify potential BoNT. Multiplex PCRs were used for