Prevalence of *Clostridium botulinum* type E in the food chain of the Lower Great Lakes

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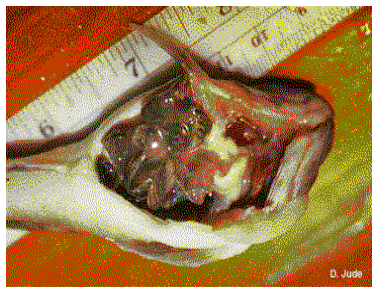
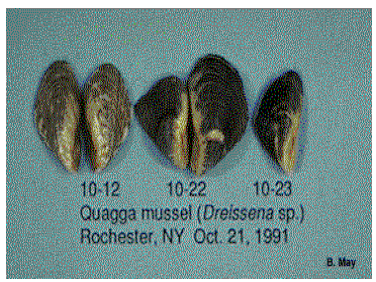
Type E botulism in the food chain?





- In our present project and we are attempting to determine the prevalence of *Clostridium botulinum* type E in lower food chain organisms.
- We want to know how these organisms may contribute to the dissemination of type E botulinum toxin to the Lower Great Lakes' fish and waterfowl.
- The first part of this effort involves looking for *Clostridium botulinum* type E in the sediments of our study areas.

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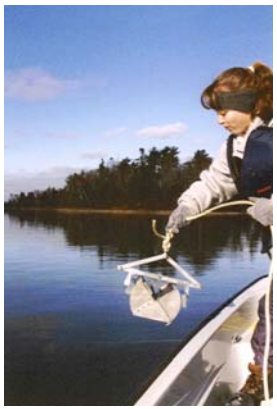
2004 Sediment collections



New York State Police Dive Team

- With the help of our NYSDEC and USFWS colleagues we have collected sediments with either petite ponar grabs or divers from near shore waters of both Lake Erie and Lake Ontario during the spring, summer, and fall of 2004.
- Ten collection sites, five shallow and five deep, were established on each lake and included those locations where documented or suspect outbreaks of botulism have occurred on Lake Erie and Lake Ontario.
- When possible, triplicate samples were taken at each site.

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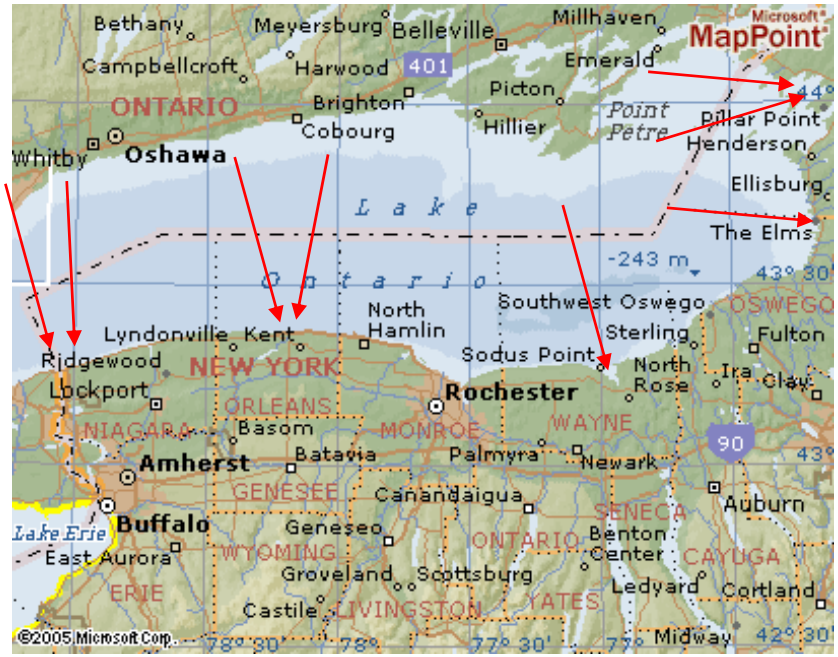
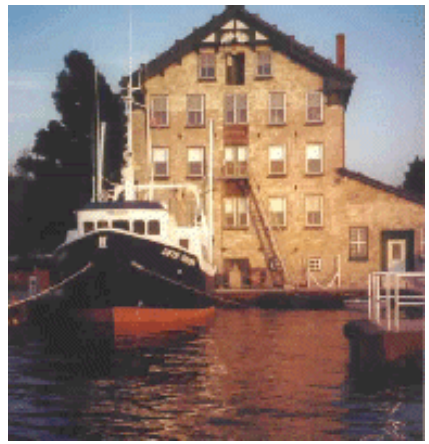
Lake Ontario sample locations





- Our collections from Lake Ontario included sites east and west of the mouth of the Niagara River, two sites near the mouth of Orchard Creek, Sodus Bay, North Sandy Pond, and two sites in Chaumont Bay. A total of 72 sediment samples and 16 quagga mussel samples were collected from May through October of 2004.

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Lake Erie sample locations

- Our collections from Lake Erie included one site each near Sturgeon Pt., Battery Pt., and Gratiot Pt.; four sites near Dunkirk Harbor, as well as sites off of Van Buren Point (three of the six freshwater drum caught near Van Buren Point were positive for type E botulism in August of 2003); and one site near Barcelona. A total of 70 sediment samples and 31 quagga mussel samples were collected from May through October.

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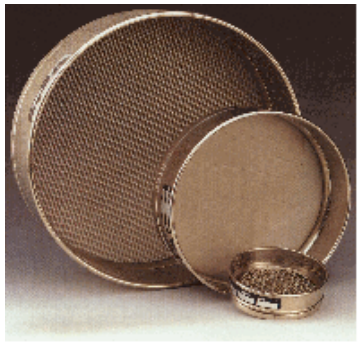
Benthic invertebrate collections



Infra Scientific Ltd

Co-operative Education Program
University of Victoria

- The second focus of our work is determining the prevalence of *Clostridium botulinum* Type E in benthic invertebrates.
- We are focusing on quagga mussels, as they were the most commonly observed food item in the stomachs of QPCR positive freshwater drum. Quagga mussels are also a favorite of the round goby; a fish species most commonly associated with botulinum intoxicated waterfowl and also found in the stomachs of dying drum.
- We also are sieving the paired sediment and mussel samples for resident invertebrates and freezing them for later QPCR testing. We expected a diverse assemblage of benthic invertebrates to be collected including larvae of dipterans, chironomids, mayflies, gammarus amphipods, mysids, and oligochaetes.
- The majority of sieved invertebrates in 2004 have been either chironomids or oligochaetes in our collections.

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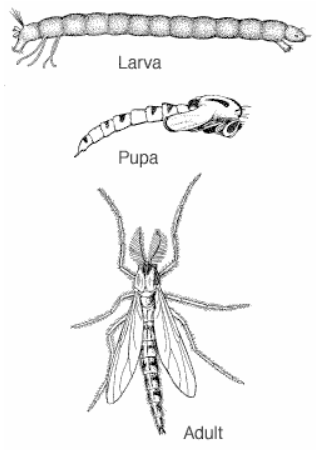
Archived sieved invertebrate samples



Darryl P. Sanders
Department of Entomology
U. Missouri

- In conversations with our collaborator, William Culligan, Region 9 NYSDEC fisheries biologist, we learned that they had been preserving sieved benthic invertebrates for the last five spring and fall sampling seasons.
- We have obtained permission to analyze one jar each from their triplicate samples for QPCR testing. The samples have been sorted and photos are being taken prior to testing.
- We are excited to determine if we can detect QPCR positive results in these archived samples.

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Prevalence of *Clostridium botulinum* type E in fish in 2004

- The third objective of our work is to determine the prevalence of *Clostridium botulinum* type E in fish from areas where *C. botulinum* type E levels are high in the sediments and invertebrates.
- From collaborations with our colleagues at SUNY Fredonia, we knew that sediments and invertebrates had tested positive for *C. botulinum* type E in the area near Dunkirk Harbor.
- So our fish collections were concentrated in that area this year. A total of 59 freshwater drum and 25 round gobies were collected in September of this year for QPCR testing.
- Two of the healthy drum collected near Dunkirk on Sept. 15th & 23rd was positive by QPCR. One of them contained round gobies in her stomach.

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Lake Erie fish mortality events in 2004

- Fish mortality events on Lake Erie in 2004, where biologists were on-hand to make a collection, were limited to two freshwater drum that were observed behaving abnormally near Dunkirk Harbor by our colleague from SUNY Fredonia, Mark Clapsadl on August 25th.
- Tissue samples were taken from both these fish and frozen for quantitative PCR analysis. These samples tested negative with the QPCR.

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Lake Ontario mortality events in 2004

- Fish and waterfowl mortalities were reported on the Canadian side of Lake Ontario during October and November. Unfortunately, few suitable samples of fish were available even though biologists from the NYSDEC were walking survey transects along New York's Lake Ontario shoreline.
- Samples of dead fish, including round gobies, smelt, alewives, smallmouth bass and several salmonids, were submitted for testing.
- Three round gobies collected on Oct. 5th & 20th near 4-mile Creek tested positive, but the gills were pale.
- The smallmouth bass collected on Nov. 1st near Porter, NY also tested positive, but the gills of this specimen were necrotic indicating that post-mortem growth of *C. botulinum* type E may have occurred.

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2004 *C. botulinum* type E QPCR results

Species	Sample Location	Collection Date	Quantity/Gram
FDrum	Dunkirk	Sept. 15, 2004	880/g IC
FDrum	Dunkirk	Sept. 23, 2004	150/g Li
RGoby	4-mile Creek	Oct. 5, 2004	1,900/g IC
RGoby	4-mile Creek	Oct. 5, 2004	34,800/g IC
RGoby	4-mile Creek	Oct. 20, 2004	83,400/g IC
SMB	Porter	Nov. 1, 2004	940,000/g IC

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2001 & 2004 Avian *C. botulinum* type E QPCR results

Species	Case Number	Sample Location	Quantity/Gram
Common Loon	01-45-19B	Lake Erie	148,000/g ACC
Common Loon	01-45-23	Lake Erie	40,700/g ACC
Common Loon	01-45-29	Lake Erie	36,200/g SC
Coot	01-45-22	Lake Erie	340/g ACC
Long Tail Duck	01-45-04F	Lake Erie	40,800/g GC
Common Loon	04-84-28	Lake Erie	11,900,000/g Li
Common Loon	04-84-32	Lake Erie	3,000,000/g Li
Common Loon	04-86-01	Lake Erie	362,000/g ACC
Common Loon	04-86-06	Lake Erie	5,900/g Li
Common Loon	04-86-09	Lake Erie	9,500,000/g ACC
Common Loon	04-86-11	Lake Erie	3,000/g ACC
Long Tail Duck	04-86-13A	Lake Erie	1,770,000/g GC
Long Tail Duck	04-86-13N	Lake Erie	11,100/g ACC

ACC = Alimentary canal contents;
GC = Gizzard contents;
Li= Liver;
SC = Stomach contents.

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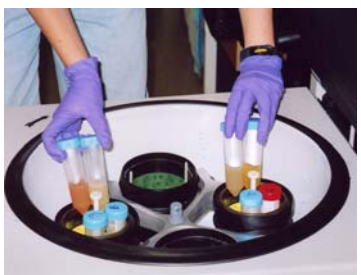
Sample processing and DNA extraction



- The traditional method for botulism diagnoses is either by anaerobic culture or the mouse bioassay.
- We have developed a molecular assay to screen samples because it is faster, safer, and more affordable.
- Fish intestinal contents and liver are processed to concentrate their DNA.
- This multi-step procedure provides purified DNA that can be assayed for the presence of the *C. botulinum* type E toxin gene.

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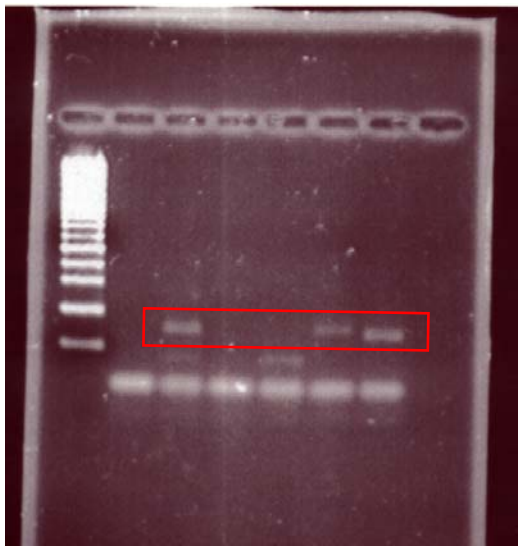
PCR versus quantitative (real-time) PCR



- After DNA is isolated, we can look for the toxin gene using a standard PCR amplification of a 139 base pair fragment to demonstrate the presence or absence of *C. botulinum* type E.
- But, quantitative (real-time) PCR will provide actual numbers of *C. botulinum* type E per gram of tissue when compared to a series of standards.
- An example of a ethidium bromide-stained gel appears on the left.

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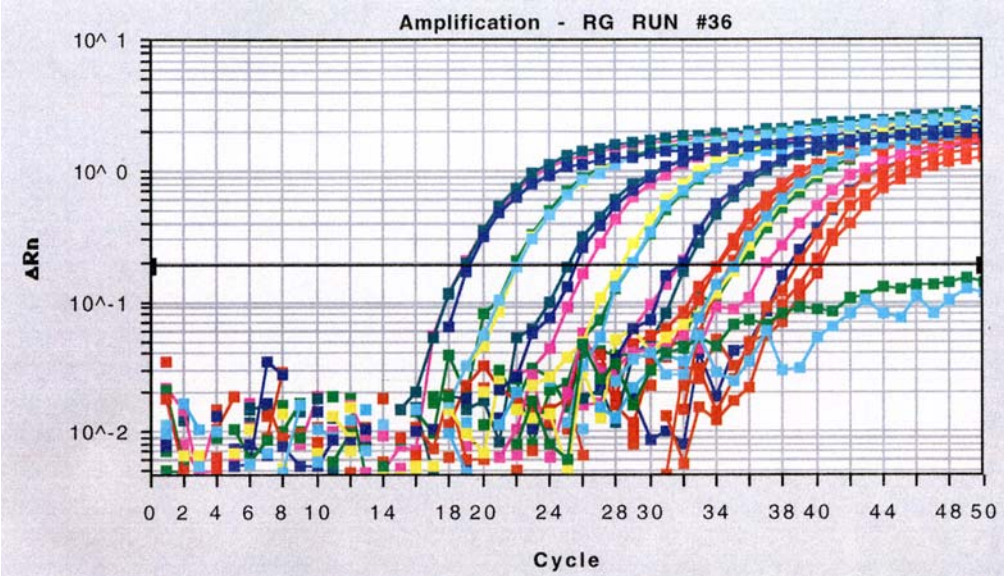
Quantitative PCR data



- Quantitative (real-time) PCR data output from ABI 7700.
- Samples tested from a freshwater drum's intestine and liver that was caught on July 11, 2002 near Dunkirk, NY appear in this plot as red squares.

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QPCR standard curve

- QPCR standard curve showing sample data (●) and standards (●) from plasmid DNA containing the 139 bp fragment of the *C. botulinum* type E toxin gene.

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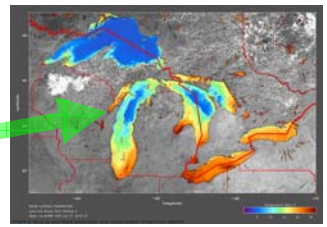
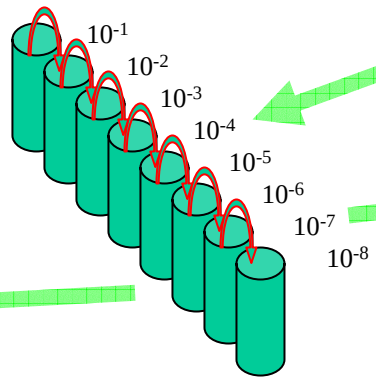
QPCR versus MPN culture & mouse bioassay

Dead lake sturgeon collected near Green Bay, WI by USFWS.

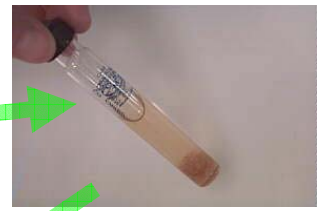


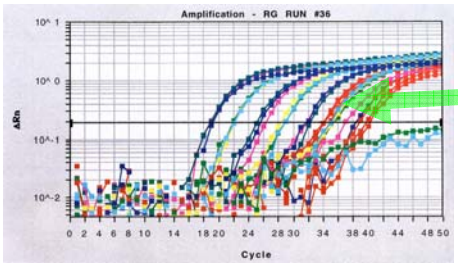
Lake sturgeon liver removed and 1 gram homogenized in peptone water. Ten-fold dilution series created.

DNA extracted from each dilution and assayed in triplicate along with quantitative standards.



Each dilution cultured in 3 tubes of chopped meat broth for 4 days in an anaerobic chamber.



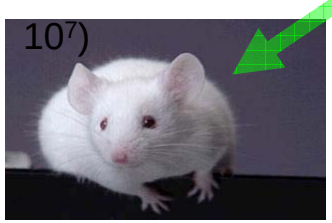


Two mice injected with each broth supernatant and observed for signs of botulism for 4 days.

The number of genome equivalents estimated by the QPCR assay was approximately **4.2 X 10^7/g** of liver (95% C.I. 2.9 X 10^7, 5.5 X 10^7).

MPN Culture & Mouse Bioassay Results

Mouse deaths per dilution					Reported MPN positive values	MPN estimate/g
10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}		
3/3	3/3	3/3	2/3	0/3	3-2-0	**9.3 X 10^6 CFU/g** (95% C.I. 1.5 X 10^6, 3.8 X 10^7)



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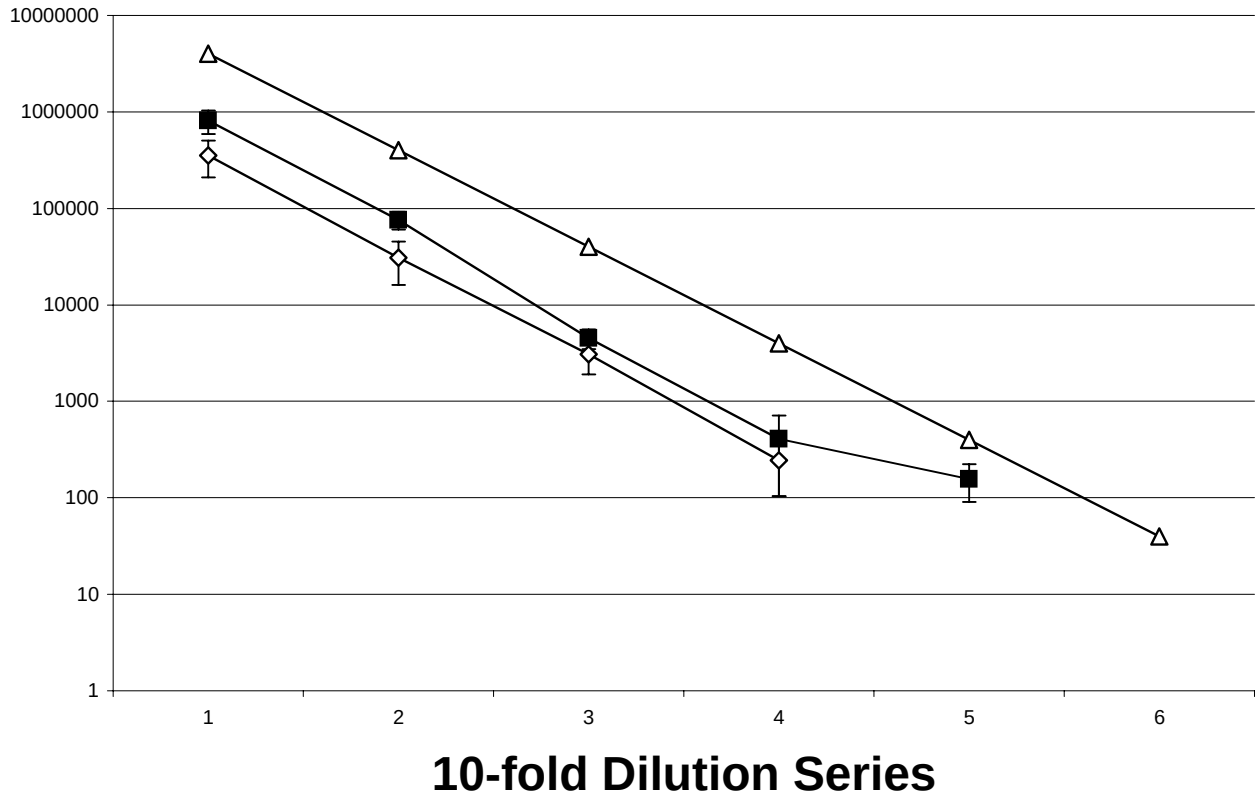
QPCR assay of plasmid-spiked freshwater drum liver and intestinal contents



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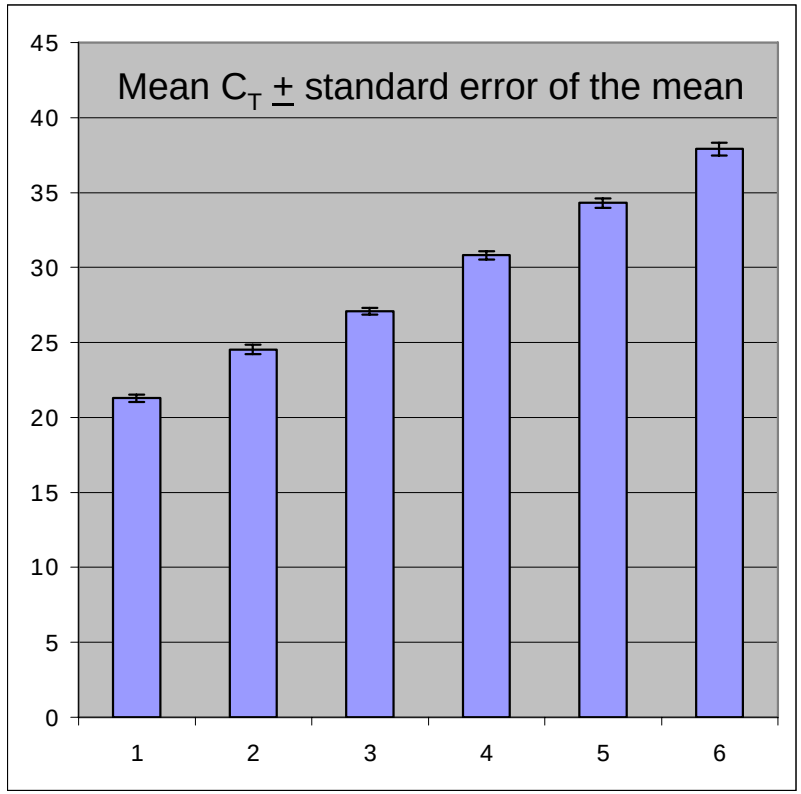
Interassay repeatability of *bont*E gene fragment quantitation by QPCR

Assay No.	C_T for number of input copies[a]					
	4,000,000[b]	400,000[c]	40,000[d]	4,000[e]	400[f]	40[g]
1[h]	22.11	25.45	27.73	31.40	35.15	38.97
2	21.03	24.43	26.92	30.26	34.19	38.58
3	20.20	23.46	26.33	29.76	33.54	36.48
4	21.80	25.35	27.61	31.29	34.77	38.90
5	21.29	24.86	26.99	31.24	34.44	37.80
6	22.47	25.91	28.27	32.83	36.12	39.58
7	20.61	24.09	26.31	30.20	33.58	36.54
8	21.16	23.71	26.65	30.42	33.72	37.44
9	20.58	23.53	26.57	30.66	33.48	37.69
10	21.62	24.70	27.37	30.60	33.96	36.82

[a]Input copy numbers of *bont*E DNA standard.



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Future research plans

- Continue to collect fish during botulism outbreaks.
- Continue to collect and test sediment, quagga mussels, and other invertebrates from outbreak areas as well as designated sites in both lakes.
- Continue the validation work on our molecular assay methods.
- Continue our collaborations with other Great Lakes researchers.

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Acknowledgements

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